APPLICATION OF PCR-MEDIATED DNA TYPING IN THE MOLECULAR EPIDEMIOLOGY OF MEDICALLY IMPORTANT MICROORGANISMS

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Belkum, Alex van
Application of PCR-mediated DNA typing in the molecular epidemiology of medically important microorganisms / Alex van Belkum ; [ill. A. van Belkum].- [S.I.: s.n.]
(Alblasserdam: Offsetdrukkerij Haveka B.V.).- I11.
Thesis Erasmus University Rotterdam.- with ref.- With summary in Dutch.
ISBN 90-9009185-8
NUGI 743
Subject headings: PCR / molecular epidemiology / medically important microorganisms.

APPLICATION OF PCR-MEDIATED DNA TYPING IN THE MOLECULAR EPIDEMIOLOGY OF MEDICALLY IMPORTANT MICROORGANISMS

TOEPASSING VAN DNA TYPERING MET DE PCR IN DE MOLECULAIRE EPIDEMIOLOGIE VAN MEDISCH RELEVANTE MICROORGANISMEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF.DR. P.M.C. AKKERMANS M.A.
EN VOLGENS HET BESLUIT VAN HET COLLEGE VOOR PROMOTIES
DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP
WOENSDAG 8 MEI 1996 OM 11.45 UUR

DOOR
ALEX VAN BELKUM
GEBOREN TE WARMOND

PROMOTIECOMMISSIE

PROMOTOR:	PROF.DR. H.A. VERBRUGH
OVERIGE LEDEN:	PROF.DR. A.D.M.E. OSTERHAUS
	PROF.DR. H.J. NEIJENS
	PROF.DR. M.J. STRUELENS

The studies presented in this thesis were performed at the Department of Molecular Biology, Diagnostic Center SSDZ, Reinier de Graaf Gasthuis, Delft, the Netherlands; and at the Department of Bacteriology, University Hospital Dijkzigt, Rotterdam, the Netherlands.

Financial support was obtained from HT Biotechnology (Cambridge, United Kingdom), Roche Diagnostic Systems Benelux (Brussels, Belgium), Amersham Nederland B.V. ('s-Hertogenbosch, the Netherlands) and Bio-Rad Laboratories B.V. (Veenendaal, the Netherlands).

AIR

CONTENTS

SCOPE OF THE THESIS

This thesis describes the development, application and validation of the newer DNA analysis techniques within the field of microbiological epidemiology. Emphasis is placed on the use of the polymerase chain reaction (PCR), a test-tube technique enabling the amplification of (parts of) DNA molecules to enormous amounts. By comparing the genomes of microbes, insight in the mode of dissemination of given microorganisms can be obtained. Besides, this type of laboratory procedures also allows evolutionary studies, highlighting genomic variability perse.

In the first chapter the technology will be introduced and the function of molecular typing within a microbiological laboratory will be explained. Subsequently, present day literature describing the application of PCR in microbial epidemiology will be summarised (chapter II). Chapters III and IV provide examples of the novel PCR approaches for molecular tracking of the protozoan parasites *Naegleria* spp. and *Giardia duodenalis*. The fact that the application of these techniques is not restricted to free-living amoebae and intestinal parasites is demonstrated in chapters V to VIII. Monitoring spread and persistence of fungal pathogens is performed, indicating the clinical relevance of genetic screening of a diversity of fungal agents, pathogens that went through a steep rise in clinical incidence during the past ten years. The last four chapters (IX to XII) describe the technical possibilities for and the clinical implications of molecular epidemiological studies on (methicillin-resistant) *Staphylococcus aureus*. Finally, chapter XIII integrates data presented in the previous chapters and gives a summary of current knowhow and sketches future developments.

Adapted from: Zentralbl. Bakteriol., in press.

CHAPTER I
CURRENT TRENDS IN TYPING OF BACTERIAL STRAINS FOR MEDICAL PURPOSES

Alex van Belkum

University Hospital Dijkzigt, Department of Bacteriology, Institute of Clinical Microbiology and Antimicrobial Therapy, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, the Netherlands. Tel: 31-10-4635813; Fax: 31-10-4633875; E-mail: vanbelkum@bacl.azr.nl.

Establishing (non)identity between independently isolated strains of a microbial species can be of major concern to the hospital epidemiologist. During times of ongoing nosocomial outbreaks, comparative analysis may reveal whether or not additional preventive measures have to be implemented and the end of outbreaks, although already preliminary defined by a decline in infection incidence, may be determined unequivocally. For the detection of differences between microbial "individuals", the clinical microbiologists has the disposal of a multitude of technological instruments. These procedures all have their specific merits and drawbacks (7), but depending on parameters like scale of application, availability of equipment, experimental expertise of analytical personnel, reproducibility and resolving power, a procedure suited for application in any clinical environment can be chosen. In general, however, the genotypic procedures, characterising microbial DNA polymorphisms, are considered to be preferable in comparison with the phenotyping strategies. These genetic procedures display enhanced resolution and superior reliability and reproducibility (9). Basic to this difference in quality is that phenotypic effects are predominantly the consequence of genetic variation, whereas genetic change does not always result in easily detectable phenotypic alterations.

Consequently, most differences between microbial strains are determined by differences in genomic nucleotide sequence or the acquisition, multiplication or loss of epi- or intrachromosomal elements like bacteriophages, insertion sequences or transposons and plasmids. This latter class of major genetic variables is relatively easy to document experimentally. Plasmid profiling for instance requires little experimental expertise and can be very informative with respect to the phylogenetic relatedness of given bacterial strains. Moreover, acquisition of an additional, relatively large genomic component like a phage or a plasmid may confer novel biologic characteristics like resistance against antimicrobial therapeutics which can simplify epidemiological monitoring. Small genetic changes within the genomic DNA molecule itself are more randomly dispersed and may differ in a location-dependant fashion. Since in a single DNA molecule multiple functions are combined (e.g. to effect genome replication, to segregate and to provide the nucleic acid sequences required for production of certain gene products), mutations do not occur

at random because of functional and structural constraints (4). When mutation occurs, it can be induced environmentally, for instance as the result of chemical or radiation-mediated DNA damage. Although bacteria are capable of DNA repair, not all mutations may be restored. DNA can also alter during erroneous steps in the replication process, for instance due to mistaken incorporation of certain nucleotides or, more directed, by mechanisms like slipped strand replication (6). It has been demonstrated experimentally that for *Escherichia coli* the divergence as measured among strains that delineate from a unique parent strain can be largely attributed to the absence or presence of prophages or artifacts which are most probably related to inefficient termination of replication (1).

Whatever the nature of the inflicted DNA change, the quality of a molecular typing procedure depends on its ability to scan an entire genome in such a way that adequate visualisation of genetic change is achieved. All DNA typing procedures, however, have their practical and theoretical limitations in this respect. When assaying changes in restriction enzyme recognition sites (restriction fragment length polymorphism (RFLP) analysis), one looks at a very restricted portion of the genome which, in addition, is of a single universal nucleotide motif. An average recognition sequence for instance occurs in a very limited and disperse fraction of the entire genome, assuming random order in the DNA chain. Using this approach, a DNA molecule is scanned only partially. This implies that randomly dispersed pointmutations will be hard to detect among closely related strains. It has to be emphasised, however, that detection of minor changes may not be very relevant to establishing non-clonality. Success in the detection of RFLP among strains implies that, besides the detected change in the recognition site involved, multiple mutational events must have occurred. Fortunately, including novel genetic elements into a genome will generally result in the acquisition of new restriction sites of the required type.

Different technical approaches enable the detection of differences in micro- versus macro-restriction fragments. Currently, pulsed field gel electrophoresis is considered one of the most promising techniques in molecular epidemiology (2). However, precise validation studies, comparing the efficacy of the procedures that are applicable for molecular typing of a given microbe, have hardly been performed.

Major present-day research efforts in microbial typing should address the validation and standardisation of molecular typing procedures. Due to enlargement of the (generally nucleic acid mediated) technological possibilities detailed comparative typing studies are urgently required. What are the theoretical and experimental requirements of the ideal typing protocol? For answering this question several typing aspects need highlighting. Ideally, the optimal system should have a discriminatory power amounting to 100% when unrelated strains are concerned. On the other hand, it should still be possible to calculate overall resemblance in an adequate manner. In contrast, detection of minimal differences between otherwise clonally related microorganisms should not confuse the analysis. This is of course also determined by the intrinsic genetic flexibility of the microbial study

object. Bacterial genomes containing large numbers of repetitive motifs or bacteria having the capability to acquire DNA by natural transformation may be genetically different, although clonally clearly related (3). Little is known on the short-term evolution of genomic DNA molecules and its consequences for the results obtained by molecular typing.

The most extensive typing system validation yet has been performed for *Staphylococcus aureus* (9, 10). Overall, the DNA-based techniques and immunoblotting were most effective in grouping epidemiologically-linked strains. This was, however, accompanied by a lack of discrimination among several of the non-outbreak strains, thereby exposing insufficient discriminatory power in some instances. Although biotyping seemed to produce to many subtypes, no single typing technique was overtly superior to any of the others. A major problem that was encountered in the multicentered comparison of a single typing technique (arbitrary primed (AP) PCR analysis, see reference 10) was the lack of reproducibility of data obtained in different laboratories.

Apparently, and that is a characteristic of most typing schedules, there is a clear need for improvement of inter-laboratory standardisation. This immediately raises the question whether or not typing should be performed in specialised, centralised facilities only. It is felt that this is dependent on the epidemiological question-to-be-studied. In case of local and sudden increases in infection incidence, typing data need to be generated at high speed to optimally serve the clinician. The procedures most suited for this, are also the ones that may be hard to reproduce between laboratories. In these cases, simple techniques allowing the swift generation of molecular pedigrees for collections of microbial isolates should be employed in a decentralised fashion. For more generalised, longitudinal and nationwide screening of pathogens there is still a clear need for centralised facilities. By concentrating all strains and optimised technologies in a single institute, accurate analysis of the dispersal of clones can be performed.

It is thus suggested to imply two types of surveillance strategies. Firstly, within a given hospital, expertise enabling high speed action upon suspicion of outbreaks should be available, whereas for large-scale screening the know-how of centralised, national institutions is essential. When recent literature is scanned, a clear shift in the number of decentralised typing studies can already be documented. Furthermore, it has been clearly demonstrated that epidemiological data obtained by the application of a single typing technique should be interpreted with caution (3, 9, 10). Preferentially, data should be corroborated by applying an additional typing scheme. The suggestion that a single procedure, which theoretically is universally applicable, should be considered with care only (5).

REFERENCES

1. **Brikun, I., K. Suziedelis and D.E. Berg** (1994) DNA sequence divergence among derivatives of *Escherichia coli* K-12 detected by PCR based RAPD DNA

fingerprinting. J. Bacteriol. **176**: 1673-1682.

2. **Goering, R.V** (1993) Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed field gel electrophoresis. Infect. Control Hosp. Epid. **14**: 595-600.
3. **Hermans, P.W.M., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum and R. de Groot** (1995) Molecular typing of *Streptococcus pneumoniae* strains with five different DNA fingerprinting techniques: a comparative study. J. Clin. Microbiol. **33**: 1606-1612.
4. **Karlin, S., I. Ladunga and B.E. Blaisdell** (1994) Heterogeneity of genomes: measures and values. Proc. Natl. Acad. Sci. USA **91**: 12837-12841.
5. **Kostman, J.R., M.B. Alden, M. Mair, T.D. Edlind, J.J. LiPuma and T.L. Stull** (1995) A universal approach to bacterial molecular epidemiology by polymerase chain reaction ribotyping. J. Infect. Dis. **171**: 204-208.
6. **Kunkel, T.A** (1993) Slippery DNA and diseases. Nature **365**: 207-208.
7. **Maslow, J.N., M.E. Mulligan and R.D. Arbeit** (1993) Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. Clin. Infect. Dis. **17**: 153-164.
8. **Pitt, T.L** (1994) Bacterial typing systems: the way ahead. J. Med. Microbiol. **40**: 1-2.
9. **Tenover, F.C., R. Arbeit, G. Archer, J. Biddle, S. Byrne, R. Goering, G. Hancock, G.A. Hebert, B. Hill, R. Hollis, W.R. Jarvis, B. Kreiswirth, W. Eisner, J. Maslow, L.K. McDougal, J.M. Miller, M.E. Mulligan and M. Pfaller** (1994) Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. J. Clin. Microbiol. **32**: 407-415.
10. **Van Belkum, A., J. Kluytmans, W. van Leeuwen, R. Bax, W.G.V. Quint, E. Peters, A. Fluit, C. Vandenbroucke-Grauls, A. van den Brule, H. Koeleman, W. Melchers, J.F.G.M. Meis, A. Elaichouni, M. Vaneechoutte, F. Moonens, N. Maes, M. Struelens, F. Tenover and H.A. Verbrugh** (1995) Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. **33**: 1537-1547.

Adapted from: Clin. Microbiol. Rev. 1994;7:174-184

CHAPTER II

DNA FINGERPRINTING OF MEDICALLY IMPORTANT MICROORGANISMS BY USE OF PCR: A LITERATURE REVIEW

Alex van Belkum

University Hospital Dijkzigt, Department of Bacteriology, Institute of Clinical Microbiology and Antimicrobial Therapy, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, the Netherlands. Tel: 31-10-4635813; fax: 31-10-4633875; E-mail: vanbelkum@bacl.azr.nl

---

SUMMARY

Selected segments of any DNA molecule can be amplified exponentially by the polymerase chain reaction (PCR). This technique provides a powerful tool to detect and identify minimal numbers of microorganisms. PCR is applicable both in diagnosis, as in epidemiology. By amplification of hypervariable DNA domains, differences can be detected even among closely related strains. PCR fingerprinting is a valuable tool for medical microbiologists, epidemiologists and microbial taxonomists. The current state of PCR-mediated genotyping is reviewed, including a comparison with conventional molecular typing methods. Because of its speed and versatility, PCR fingerprinting will play an important role in microbial genetics, epidemiology, and systematics.

INTRODUCTION

Typing assays are necessary because several species of microorganisms share overlapping niches or thrive under identical environmental conditions. This need has led to the development of a large array of molecular techniques that can be used to determine identity or nonidentity of living organisms and that enabled detailed comparisons of large collections of organisms. The most powerful procedures are based on genetic characterization. Once a species can be adequately recognised, studies on its ecological behaviour and spread can be initiated.

In medicine, the species identification of a microorganism is important for prevention, diagnosis, and treatment of infectious diseases. Identification and additional typing can be done on the basis of the growth characteristics of an organism on a selective medium, the recognition of microbial antigens by monoclonal or polyclonal antibodies, biochemical characteristics, antibiotic susceptibility, susceptibility to infection by bacteriophages, and general features such as odour and colony form. Combination of all data, including those gathered by the use of high-resolution molecular techniques, can lead to unequivocal identification and, in some cases, can even discriminate among isolates of a given species. These data can be used for epidemiologic purposes. Determination of unique characteristics of a microorganism allows study of colonization or cross-infection and enables the establishment of phylogenetic relationships.

In this paper the methods used for genetic typing of eukaryotic and prokaryotic microorganisms will be surveyed with emphasis on a recently developed DNA amplification mediated procedure. The future role of this type of amplification- mediated genetic analyses in microbiology will be discussed.

CURRENT MICROBIAL TYPING METHODS

Methods used for discrimination of genera, species, and isolates can be divided into phenotypic and genetic procedures. Phenotypic procedures take advantage of biochemical, physiological, and biological phenomena, whereas the genetic procedures aim to detect polymorphisms at the level of nucleic acids or at allelic variation at the level of enzymes.

Phenotyping: Classical microbiological diagnosis is based on microbial morphology and staining properties and the ability of a microbial species to grow under a given set of environmental conditions defined, for instance by temperature, oxygen dependence, and osmolarity. The need for certain nutrients is also an important parameter. Biochemical tests alone usually allow species identification, but can also help distinguish among strains of organisms. Antimicrobial susceptibility assays are used mainly for the selection of appropriate therapy, but, again, may also help to discriminate among strains, although the test sensitivity is limited, especially when the organisms are highly resistant or highly susceptible (72). A number of other highly specific phenotyping assays such as phage typing have been developed (33). These biological tests are laborious to perform and require sophisticated reagents which are often not commercially available. The methods, however, can be used for adequate epidemiological analyses of various organisms. *Staphylococcus aureus* is a well-known example of a bacterium that can be effectively phage typed. Alternatively, protein analysis can be used to delineate the origin of a microbial strain and to establish relationships among isolates. Techniques such as whole-cell protein profiling, outer membrane profiling, isozyme electrophoresis, and various immunoblotting techniques are frequently used in research settings (28).

Phenotypic procedures are generally not meant for discrimination among strains of different species. Although, for instance, several monoclonal antibodies have been described as type specific, they often display cross-reactivity with additional antigens (90). Also, quite frequently certain groups of isolates remain untypeable by these strain-specific monoclonal antibodies, mostly for unknown reasons. It is for this and other reasons that genetic procedures are preferred when strain differentiation is required.

Genotyping: That the genome of each individual is unique is basic to all DNA analyses aimed at identification. In organisms that reproduce sexually, differences occur because the offspring inherits different alleles from either parent. However, genetic differences can also be demonstrated between individual isolates of an asexually reproducing species. These differences trace back to intrinsic capacities of the genetic material present in these organisms. For instance, an organism such as the malaria parasite,

Plasmodium spp., harbours a genome of approximately 15 x 10^6 to 20 x 10^6 base pairs divided among 15 chromosomes (99). Differences in the length of homologous chromosomes from different parasite isolates going through asexual reproduction only can be documented (42). These differences arise through various mechanisms, mainly DNA recombination (107). More subtle changes also occur. For example, repetitive DNA sequences can give rise to mobile DNA fragments and to more localisd DNA polymorphisms through replication slippage (44, 108). Also DNA polymerases tend to accidentally misincorporate wrongly positioned base residues (54), which may lead to point mutations.

Genetic variation can be documented by different molecular biological techniques. For instance, chromosomal length variation can be determined directly by electrophoretic separation of entire chromosomes in agarose gel-based systems (80, 87). Variation due to mobile or repetitive DNA elements can be traced by specific DNA probes, often in combination with a restriction enzyme treatment and Southern blotting (9, 17). General DNA probes, e.g. those for rRNA genes (55, 93, 65, 117) can be used for screening. The most detailed analyses can be performed by direct nucleotide sequence analysis of specific regions in the genome (25, 38, 122). This approach, however, is technically demanding and is not yet within direct reach of the clinical laboratory.

Genetic typing assays also have drawbacks. In general, these procedures require relatively large amounts of high quality DNA or RNA and a high degree of technical skill. Therefore, simple procedures in which small amounts of relatively impure nucleic acid is required have been developed.

NUCLEIC ACID AMPLIFICATION IN MEDICAL MICROBIOLOGY

Several techniques for enzymatic amplification of nucleic acid sequences have been developed in the past decades, which are now being evaluated in medical microbiology. The ligase chain reaction (LCR), for instance, can be used for sensitive detection of DNA point mutations (4), whereas the Qß system, taking advantage of the hyperactive RNA-dependent RNA polymerase from the bacteriophage Qß (48, 56), provides a high-speed RNA amplification assay. The nucleic acid sequence-based amplification (NASBA) assay, also known as self-sustained sequence replication (3SR) can be performed under isothermal conditions and is useful for detection of clinically relevant pathogens (22, 47). The polymerase chain reaction (PCR) is the prototype nucleic acid amplification method and it has been extensively evaluated (6, 69, 70, 115). This technique has evolved from a laborious and relatively insensitive assay into an extremely sensitive and highly flexible procedure. The discovery of thermotolerant DNA polymerases (79) and the development of automated PCR processors have facilitated the introduction of PCR into the diagnostic laboratory and have led to an exponential increase in the number of PCR applications. DNA from any source, including insects trapped in amber millions of years ago or archival mummy tissue, can be amplified (7, 67). The diagnostic applications of PCR are the most important in clinical microbiology. Several dozens of highly sensitive and

specific assays have been developed for the detection of clinically important microbial pathogens (14, 49, 74, 86, 109). Fastidious organisms such as fungi (119) and mycobacteria (13), that require prolonged periods of cultivation, can now be detected within substantially shorter periods. PCR has also been adapted for the direct comparison of homologous microbial DNA molecules, in analogy with DNA fingerprinting procedures that are accepted in forensic science (45).

PRINCIPLES OF PCR FINGERPRINTING

The basis of PCR fingerprinting is the amplification of polymorphic DNA through specific selection of primer annealing sites. Either constant primer sites bridge a single variable sequence domain (Figure 1B) or primers detect consensus sequences with variable distribution in the DNA (Figure 1A). Differences in the distance between primer-binding sites or existence of these sites lead to synthesis of amplified DNA fragments (amplimers) which differ in length. These differences can be detected by simple procedures such as gel electrophoresis or chromatography. PCR fingerprinting has been described by different names and accompanying abbreviations. Terms such as amplification fragment length polymorphism (AFLP), DNA amplification fingerprinting (DAF), arbitrarily primed PCR (AP PCR), interrepeat PCR (IR PCR), or random amplification of polymorphic DNA (RAPD) are used indiscriminately. The applications of PCR fingerprinting in forensics and diagnostic microbiology will be discussed.

Fingerprinting in forensics: PCR fingerprinting for forensic applications usually relies on the strategy depicted in Figure 1A (45). Several variable loci are amplified and direct inspection of amplimers enables identification of kinship or common source of biological material. Major targets for these assays are the variable number of tandem repeat (VNTR) loci. VNTRs can display extensive variations in length and form a rich source of potentially useful genetic markers, not only for identification of individuals but also for localizing "disease" genes or mapping other genetic traits (84). Since the first time PCR fingerprinting results were used in court (1986, in the case Pennsylvania versus Pestinikis), PCR tests have been used as evidence in criminal cases (2, 37). There is an ongoing discussion on the reliability of PCR mediated-DNA profiling. The main question is whether the alleles that are studied are sufficiently polymorphic to warrant reliable conclusions for all ethnic subgroups. To provide these data, DNA screening programs involving large groups of people are being performed (18, 27).

Genetic variation in bacteria, yeasts and parasites: In studies with microorganisms, variable regions such as those found in higher eukaryotes have been sought. Fungal DNA, for instance, appears to contain several DNA repeats similar to those of higher eukaryotes, and similar sequence motifs have been identified in several protozoan species (97, 98). Also, the bacterial genome harbours repetitive sequences that can be used for DNA-typing (58, 107). The characteristics of these sequences are their restricted length and their widespread occurrence. Prokaryotic repetitive motifs occur throughout

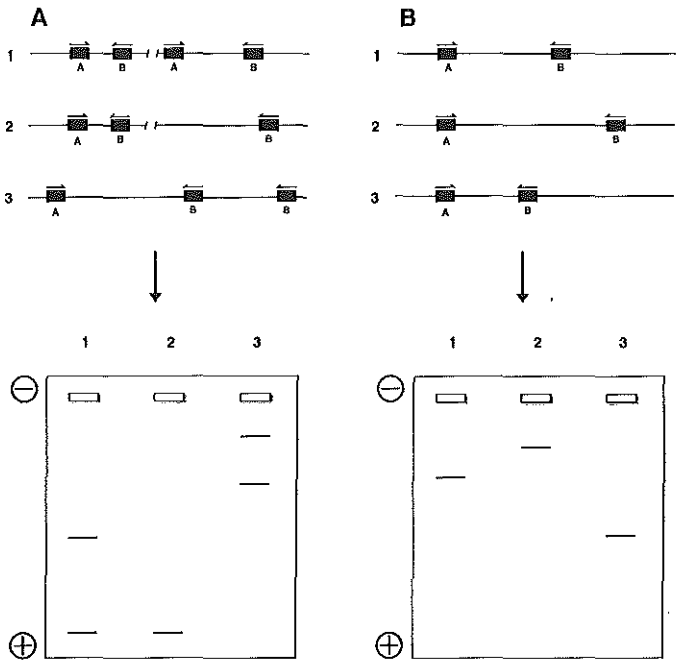


Figure 1 PCR mediated DNA fingerprinting: two variants. Panel B describes the results of DNA amplification using primers that anneal to constant binding sites, which span a variable segment of DNA. In this example DNA 1 and 3 could be deleted as compared to DNA 2. Otherwise, DNA 1 and 2 could also harbor insertions lacking in DNA 3. The upper part of the panel gives the theoretical background for the electropherogram shown below. Panel A examplifies primer binding site variation. Cases 2 and 3 lack a site present in DNA 1. This results in disappearance of a band in the electropherogram. In this example multiple primers included in a single PCR reaction may enhance the number of polymorphic sites that can possibly be detected.

the entire genome but rarely within genes. Repeats were initially discovered in *Escherichia coli* and *Salmonella typhimurium* (36, 40) but were later found in several other bacterial species (Table 1). Little is known about the functions of these repeats. They maybe involved in the regulation of transcription or translation or the maintenance of chromosomal organization (58). The presence of these sequences can be exploited in genetic manipulation of prokaryote genomes (94). Prokaryote repeats can be considered

Table 1 Repeat motifs in bacteria

name	length	organism	ref.
		(bp)	
Repetitive extragenic palindrome (REP)	38	*Escherichia coli* *Salmonella typhimurium*	36, 40
Enterobacterial repetitive intergenic consensus (ERIC)	124-127	*Escherichia coli* *Salmonella typhimurium*	41, 83
NgREP	26	*Neisseria gonorrhoeae*	21
DrREP	150-192	*Deinococcus radiodurans*	53
MxREP	87	*Myxococcus xanthus*	32
REPMP1	300	*Mycoplasma pneumonia*	114
SDC1	400	*Mycoplasma pneumonia*	20

simplified forms of eukaryotic VNTRs. When PCR primers similar to those used for eukaryotic VNTRs are designed, the DNA amplification process results in the generation of highly specific and reproducible DNA fingerprints that enable discrimination between isolates of a single bacterial species (see below). Apparently, minimal genetic differences can be determined through this relatively simple technique.

In addition to repeat-based genetic variation, DNA polymorphisms can be detected by using short DNA oligonucleotides (approximately 10 nucleotides in length) with a random sequence as primer. Non stringent annealing temperatures in the PCR also allow detection of random genetic variation. A combination of these two experimental conditions, called RAPD analysis, was first used to demonstrate strain variability in inbred mouse lines (110, 111, 116) and has recently been adapted for the discrimination between RNA (cDNA) populations as well (112). This approach also found widespread use in establishing microbial variance (reviewed in reference 5). In addition to targeting repetitive or random sequences, aiming for other sequences occurring at multiple sites in a genome may also be effective. For example, tRNA sequences (82), genes coding for specific protein stuctures, or DNA segments regulating gene expression are interesting targets (8). This fingerprinting approach has been named motif sequence-tagged PCR.

TECHNICAL ASPECTS OF PCR FINGERPRINTING

When setting up a PCR fingerprinting facility, all usual precautions to prevent contamination must be taken (50). PCR fingerprinting, however, is less vulnerable to contamination than is PCR used for detection, since relatively large amounts of pure template DNA are used. A clear description of an optimal system has recently been given

by Bassam et al. (5). These authors varied all parameters that can influence the efficiency of PCR fingerprinting. After the optimal Mg^{2+} concentration was determined, the effectiveness of different amounts of different Taq polymerases was compared. The exonuclease-deficient Stoffel fragment of the Taq polymerase (51) generated fragments that appeared most diverse in length. This modified, enzyme also was most efficient in

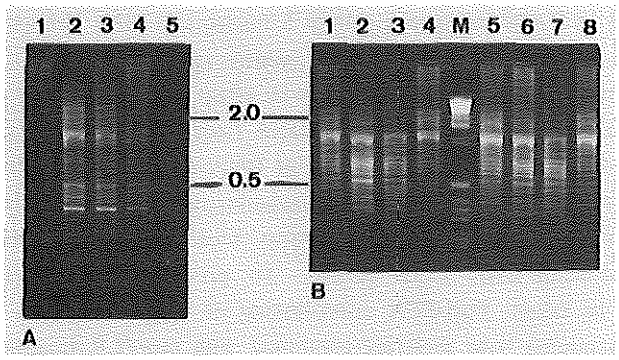


Figure 2 DNA template concentration dependence and influence of primer purity on PCR mediated DNA fingerprinting of *G. duodenalis* strains. **(A).** DNA from the *G. duodenalis* Nij-2 strain was amplified with arbitrary primer BG2 (5'TACATTCGAG-GACCCCTAAGTG). DNA was included in the PCR in different amounts: lane 1 shows the result of a negative control sample containing no parasite DNA. Samples 2 to 5 contain decreasing amounts of *G. duodenalis* DNA ranging from 50 ng, 5 ng, 0.5 ng to 50 pg (lanes 2 to 5, respectively). Although staining intensity decreases from left to right no major changes in the DNA banding pattern became apparent. Only in the low molecular weight range (100 basepairs) do small changes occur. This implies that the reproducibility of PCR fingerprinting is not strongly affected by the template DNA concentration. **(B).** DNA from the *G. duodenalis* AMC-5 strain was amplified by using crude and purified fractions of two independently synthesised batches of primer BG2. Template DNA was added in a constant amount of 5 ng. Lanes 1 to 4 displays the results obtained with crude primer (lane 1), reversed phase cartridge (Pharmacia) purified primer (lane 2), and two HPLC-purified fractions (lanes 3 and 4). The difference between lanes 3 and 4 lies in the fact that in lane 3, the primer was chemically deprotected after HPLC, whereas the primer used to generate the results shown in lane 4 was deprotected before HPLC. Lanes 5 to 8 display the results of identical experiments performed with the independently synthesised primer batch. It can be concluded that the PCR fingerprints do not vary strongly with the purity of the primer used. Second, and more important, if primer batches are processed in an identical fashion (crude, RPC, HPLC), fingerprints are identical (lanes 1 and 5, 2 and 6, 3 and 7, and 4 and 7). This reproducibility indicates the usefulness of PCR fingerprinting in long-term longitudinal screening programs.

DNA synthesis. No batch-to-batch variation between enzyme preparations was detected. Use of ten to twenty picograms of DNA led to recognizable DNA fingerprints, but increasing the amount to more than 1 ng greatly improved both resolution and reproducibility (Figure 2). An important component for the genotyping reaction is the DNA oligonucleotide primer. In general, the primer concentration should be above 0.3 μM; higher concentrations (up to 9 μM) do not significantly change the DNA patterns. Also, use of primers of appropriate length is essential. It has been shown that the optimal length for primers used in RAPD is approximately 8 nucleotides (15). Primers longer than 10 nucleotides have less discriminatory power, which again is strongly dependent on the annealing temperature. Primer purity also has an effect; only identically processed and/or purified primer batches give rise to identical DNA fingerprints (Figure 2).

An important practical disadvantage of DNA amplification is that all amplimers deriving from previous assays can act as contaminating templates in later experiments. PCR fingerprinting studies frequently involve identical primers, which makes the contamination problem even more serious. To prevent cross-contamination, several precautions can be taken. Post-PCR cross-linking of amplified DNA, incorporation of desoxyuridine-containing base moieties combined with pre-PCR treatment by uracil N-glycosylase and use of post PCR alkaline hydrolysis of products synthesised by primers containing 3' ribose residues apparently are effective for the elimination of carry-over contamination, especially when amplimers exceed 100 base pairs (30, 78). However, in our experience it is sufficient to implement the suggestions of Kwok et al. (50) i.e., keeping amplimers physically separated from template DNA; this guarantees reliable PCR fingerprinting studies.

PCR fingerprints are usually visualised by simple ethidium bromide staining of the electrophoretically separated DNA (in agarose or polyacrylamide) or by autoradiographic or fluorimetric detection of labelled amplimers (16). Fingerprints are recorded as banding patterns, and comparisons can be made by visual inspection. Automated screening by densitometers isnecessary when the number of fingerprints increases. Densitometry records not only peak position but also peak intensity, which may yield more quantitative data (Figure 3). Densitometric analysis programs enable phylogenetic comparisons (26, 120).

Sometimes PCR fingerprinting is hampered by the presence of inhibitors in the DNA. We found that processing DNA by a guanidinium isothiocyanate method (11) produces high quality preparations, almost independent of the DNA origin and free of contaminants that inhibit amplification. The size of the genome may be a problem. Typing of *Mycoplasma* species, for instance, which have a genome of approximately 10^6 bp without extensive repetitive DNA, is rather complex. Many primers or even primer combinations have to be evaluated to obtain adequate resolution. As another example, genetic identification of viruses is usually based on the detection of minor sequence variation, often in highly restricted portions of the genome. This has been described for

human papillomaviruses (59, 89) and the hepatitis C virus (106). The variable sequences can subsequently be identified by probe hybridization, restriction fragment length polymorphism determination, or direct sequencing. The last approach is now used more often in epidemiological studies and may replace all other genetic typing assays. The precise nucleotide order in a given region of a genome provides the most detailed information on DNA origin.

Several organisms and viruses have to be cultivated in cell lines and carefully purified to reduce contamination with host cell DNA before PCR fingerprinting. In many

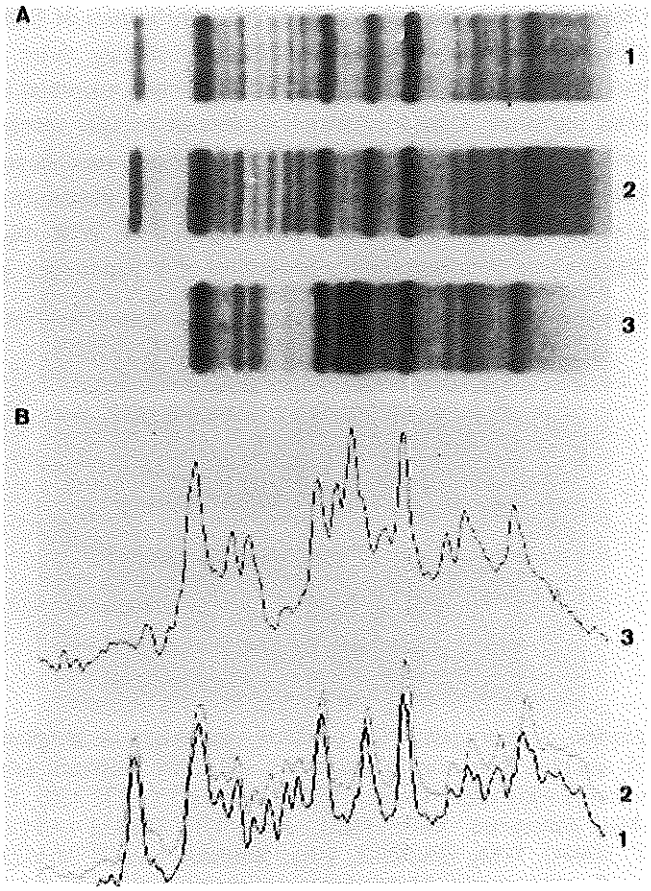


Figure 3 Densitometric scanning of PCR fingerprints. For interpretation of banding patterns (A) they are changed into transversal scans (B). This enables the study of direct overlaps in patterns and the peak height gives an impression of quantitative differences. The identity of DNA fingerprints 1 and 2 (panel A) is underscored by direct comparison of the scans derived from the electropherograms. The scans allow positioning and subsequent digitizing of peaks deriving from bands representing DNA fragments.

cases this requires complex and labour-intensive techniques. A novel strategy for purification ofmicroorganisms is provided by antigen capture assays in which specific antibodies attached to a solid support such as immunomagnetic particles are used to isolate microorganisms from complex mixtures.

PCR FINGERPRINTING IN EPIDEMIOLOGY

Genetic typing of microorganisms can provide insight into the spread and persistence of pathogens. Discrimination between coincident but independent infections and epidemics caused by a single isolate is a major concern that directly affects the preventive and hygienic measures to be implemented. Application of PCR fingerprinting can provide answers to several important questions, including those concerning the international spreading of infectious agents and clonality among microbial isolates.

Protozoan parasites: Genetic variation in several parasite species is well-documented. This variation may be programmed as in *Trypanosoma* variable surface glycoprotein (VSG) switching or be random as in the size variation of plasmodial chromosomes. Because of the major importance of DNA variation, several PCR studies have been performed. RAPD analysis of interspecies relationships of trypanosomes and *Leishmania*

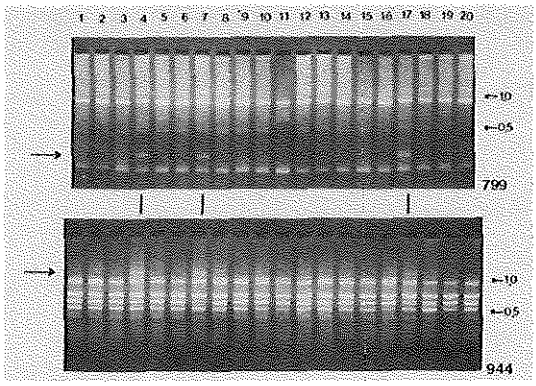


Figure 4 Comparison of interrepeat PCR products synthesised on template DNA isolated from *N. fowleri* samples from throughout the world. Two different primers aiming at repetitive DNA motifs were used. Lanes 1 to 20, isolates TY, T37 (50), F44, NA3, NHI, MCM, SW1, Mst, Enterprise, M4E, HB-1, J16/1/42E, ORAM (ATCC 30463), 124, LEEE-1 (ATCC 30894), WM, J26/50/42, PAa, Northcott (ATCC 30462), PA34 (ATCC 30468), and Lovell, respectively. Molecular mass markers (in kilobases) are indicated on the right; the arrows on the left point to additional bands that were visible only in the isolates of the New Zealand subtype (lanes containing a New Zealand subtype DNA are connected by vertical lines). By using primer 0, a single band was observed in all isolates, including those of the New Zealand subtype (data not shown). Reprinted from reference 100 with permission of the publisher. The numbers 799 and 944 encode the primers used for PCR.

species revealed a similar phylogenetic tree as determined by multilocus enzyme electrophoresis (95). The RAPD method can also be used to study species evolution, to analyze population genetics, and to identify species. By RAPD analysis, a clonal population structure for *Trypanosoma cruzi* could be further substantiated. Similar to the trypanosome situation, clonality could be deduced from a PCR fingerprinting study of species of *Naegleria*. In this particular case the PCR fingerprinting data were corroborated by protein-profiling studies (103). The different *Naegleria* species could be easily discriminated by interrepeat PCR employing simple sequence motif primers. However, 20 different *N. fowleri* isolates from diverse geographic origins were identical when two different primers were used (Figure 4). Only in three isolates from New Zealand were very minor but consistent differences detected. The intestinal parasite *Giardia duodenalis* shows a completely different picture (100). Different isolates could easily be discriminated with the same primers that showed no difference in *N. fowleri*. Even different clones from a single isolate showed specific PCR fingerprints. Apparently, the genus *Giardia* may consist of a collection of different species. Species variability was also detected among *Giardia* isolates when other variable DNA repeat motifs were amplified (76). Further investigations are required to elucidate the discrepancy between *Giardia* species and other protozoan parasites studied thus far. Finally, it would be interesting to determine whether PCR fingerprinting can be used to detect strain virulence in *Toxoplasma gondii* isolates, as can be achieved with DNA probe assays (85).

Fungi: Fungal infections are a major threat, especially to immunocompromised patient (19). To study fungal epidemiology, several techniques have been applied successfully (73). *Aspergillus* and *Candida* spp. have received widespread attention, and PCR-mediated genotyping has been used for these genera (3, 52, 57, 66). RAPD analysis appeared to be useful for species determination of several *Candida* strains. Taxonomy, confirmation of strain identity and direct epidemiological identification of strains by repeat primer PCR appeared to be possible (66). Simple sequence and telomer consensus primers could be used for the same goal. Both RAPD and interrepeat PCR (105) distinguish clinical isolates of *Aspergillus fumigatus*. *Histoplasma capsulatum* was also successfully subjected to RAPD (46, 120). The traditional criteria for typing fungi are not always clearcut: DNA fingerprinting can also be used to provide answers to taxonomic mycological questions outside the area of medical mycology. It has been shown that RAPD analysis can be used to differentiate between strains of plant-infecting fungi with different pathotypes (23). Also, the identity of what is now supposed to be the largest living organism on Earth, the fungus *Armillaria bulbosa*, has been confirmed by PCR fingerprinting (88). Phylogenetic trees constructed on the basis of restriction analysis of mitochondrial DNA from the black fungus *Hortaea wernecki* were confirmed by PCR fingerprinting studies (96).

Bacteria: Genetic typing of bacteria by PCR amplification of variable DNA stretches has been described for a large series of medically relevant species (10, 12, 24,

26, 31, 39, 43, 60, 61, 63, 64, 75, 77, 92, 113, 118, 120 and references therein). Further discussion is restricted to a limited number of species (*Staphylococcus aureus, Legionella pneumophila,* the thermotolerant *Campylobacter* species, and *Helicobacter pylori*), but all relevant aspects of microbial PCR fingerprinting will be covered.

Staphylococcus aureus: *S. aureus* frequently causes nosocomial infections, and the spread of methicillin-resistant *S. aureus* (MRSA) variants has urged implementation of reliable and efficient identification and typing techniques. The usefulness of PCR fingerprinting for *S. aureus* has been evaluated in comparison with several new molecular typing schemes. Fingerprinting has been compared with phage typing, traditionally themost frequently applied technique (101). PCR assays aiming at prokaryotic consensus repeats, arbitrary sequences, or sequences of the methicillin resistance gene complex detected nearly twice as much strain variation as did phage typing. Therefore, the PCR

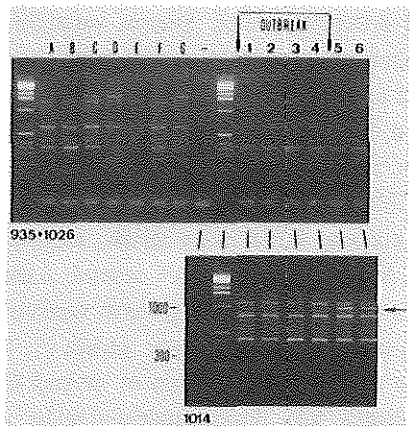


Figure 5 DNA typing of MRSA strains from diverse sources. Lanes A to G display results obtained with a set of different marker strains. Shown is the diversity in DNA fingerprints observed in a group of 48 MRSA isolates. Lanes 1 to 6 contain strains derived either from an MRSA outbreak in a geriatric nursing home (lanes 1 to 4) or from another patient (lanes 5 and 6). The outbreak samples, from which 24 different isolates were available, appear to be genetically homogeneous, as indicated by PCR-mediated genotyping with 2 primer sets. The latter combination does not enable discrimination of samples in lanes 5 and 6 or 1 to 4. Primer 1014, however, clearly differentiates the large group, which showed identical banding patterns, from the strain isolated from a patient not from the geriatric hospital (see the arrow on the right, indicating an additional band in lanes 5 and 6). The lengths of the molecular size markers (1-kb ladder; GIBCO-BRL) are indicated on the left of the lower panel, in base pairs. Reprinted from reference 98 with permission of the publisher. Numbers 935, 1026 and 1014 identify the primers used for PCR.

fingerprinting allowed detection of genetic variants in homogeneous phage groups. On the other hand, a clear overlap between phage typing and PCR typing was discovered, which underscores the validity of PCR mediated DNA typing. Strains isolated during epidemics appeared to be genetically identical (Figure 5), whereas the combination of phage plus DNA typing data enabled discrimination of more than 75% of the 48 epidemiologically closely linked isolates included in the study (101). In another recent study on *S. aureus* infection in outpatient and paediatric patients (102), similar resolution was achieved by a combination of several PCR assays. The latter study also revealed that amplified DNA fragments (amplimers) can be used as *S. aureus* isolate-specific probes (102). PCR fingerprinting of MRSA has been compared with typing by pulsed-field gel electrophoresis (81, 91). In the study by Saurnier et al. (81), RAPD analysis appeared less discriminating than PFGE because the latter technique discriminated all 26 isolates tested. RAPD with three different primers identified 25 different types in the same collection of strains. On the basis of individual PFGE or RAPD pattern homologies, similar dendrograms were constructed, indicating that different techniques lead to the same taxonomic classification. In a recent study (91), the same conclusion was drawn: clonal delineation by macrorestriction analysis showed statistically relevant concordance with with PCR genotyping.

Table 2 Result of various typing assays performed on 29 *L. pneumophila* serogroup 1 strains

Source of strains (no.)	number of different types in: PF assay 1	PF assay 2	MLEE	REA	MREA	SB
clinical isolates (10)	1	1[a]	1	1	1[a]	1
water isolates (8)	1	1	1	1	1[a]	1
unrelated sources community referred cases (4)	4	3	3	4	4	3
other hospital water (4)	4	4	4	4	4	3
NCTC 11404 NCTC 12008 NCTC 11192	3	3	3	3	3	3

Note: PF: PCR fingerprinting; MLEE: multilocus enzyme electrophoresis; REA: restriction enzyme analysis; MREA: macrorestriction enzyme analysis; SB: Southern blot RFLP with random DNA probe. [a]Minor differences were found among the fingerprints.

Legionella pneumophila: *L. pneumophila* is a pathogen that causes sporadic nosocomial outbreaks. Clinical and environmental isolates of outbreak-related *L. pneumophila* originating from a single hospital showed closely related amplimer patterns (104). Several isolates derived from the water system appeared to be identical when assayed by two different PCRs, whereas isolates from patients showed some minor deviations from each other. These differences in fingerprints were not as significant as the differences between isolates from unrelated sources, which may imply that on infection of a patient, either a strain is selected or minor genetic variance is induced by the interaction between host and bacterium. Comparisons like these may indicate that PCR fingerprinting could eventually be used for the elucidation of the genetic basis of phenotypic variability in *Legionella* spp. Also, PCR fingerprinting proved to be as effective as other typing procedures for *L. pneumophila* (Table 2). The five other test systems used were effective in discriminating NCTC strains, and all procedures documented homogeneity among related clinical and water isolates. Both PCR fingerprinting and the two types of DNA restriction analysis discriminated among other community- or hospital-referred isolates. Multilocus enzyme electrophoresis and Southern hybridization studies could not distinguish all isolates. Recent studies have revealed that other *L. pneumophila* serotypes including serologically untypeable strains can be assayed specifically by PCR fingerprinting.

Campylobacter species: Several *Campylobacter* species can be responsible for food-borne gastrointestinal diseases, which may be prevented by timely detection of contaminated sources (68). For identification of *Campylobacter* strains, RAPD analysis has been applied successfully and may even replace serotyping tests (62). A simple procedure in which bacteria were boiled and the lysate was directly introduced in the PCR vessel enabled reproducible typing of serologically nontypeable strains. PCR fingerprinting of *Campylobacter* spp. is well suited for epidemiological studies (29, 35). Several groups of isolates collected during epidemics were genetically homogeneous whereas all unrelated strains tested were clearly different. The three main pathogenic species, *C. coli*, *C. jejuni*, and *C. lari*, cannot be easily identified to species level by traditional techniques. However, PCR fingerprinting of a large number of strains of the different *Campylobacter* species showed that several amplimers were synthesised when template DNA from all strains of the three individual species was used. These amplimers were used in hybridisation studies and were shown to be species-specific (Figure 6). Even on Southern blots containing genomic DNA of the three *Campylobacter* spp., absolute species specificity was maintained. The combination of PCR fingerprinting and probe hybridization resulted in a highly specific identification and provided a first example of species test development without the prior need for DNA sequence information. This approach holds great promise for the rapid development of all types of DNA probes, which in combination with a general PCR assay, may lead to efficient and direct typing and detection procedures for many organisms. This procedure may be especially time and cost effective

when the detection and typing of multiple infectious agents in a single specimen is required.

Helicobacter pylori: *H. pylori* is suspected to be involved in several gastric diseases (71). It was one of the first pathogenic microorganisms that was investigated in great detail by RAPD fingerprinting (1). The organism appeared to be highly variable: among 64 isolates, 60 different types were found using a single primer assay. Follow-up studies revealed that failure of antimicrobial treatment or inadequate gastric ulcer resection resulted in the persistent presence of the same *H. pylori* genotype. Also, multiple genotypes could be found in a single patient. *H. pylori* studies reveal that PCR fingerprinting may be important in medical follow-up studies and in establishing the success of antibiotic therapy.

FUTURE DEVELOPMENTS

Forensic sciences and the study of infectious or genetic diseases will be among the major areas for application of PCR-mediated genotyping. PCR typing provides the potential to analyze most of the medically important organisms by a single technique. PCR fingerprinting also enables the unravelling of the genetic basis of phenotypic characteristics. The availability of isogenic microorganisms that differ in only a single feature allows PCR-mediated generation of genetic markers. Moreover, PCR genotyping facilitates the establishment of specific DNA probes. These developments will allow the combination of detection and typing of microorganisms in a single PCR. Major improvements in sample processing are also under development; these include "whole cell" PCR fingerprinting, in which crude bacterial lysates are used directly, thereby reducing the processing time and technical complexity of PCR genotyping (121). Widespread application of the technique, however, requires innovative methods for the analysis of DNA fingerprints by automated densitometry. It has to be emphasised that the PCR fingerprints should be of adequate complexity, allowing in-depth comparison. Finally, typing data obtained by DNA analysis should always be considered together with epidemiological information, since only this combination will enable unbiased evaluation of the spread or genetic variation of microorganisms.

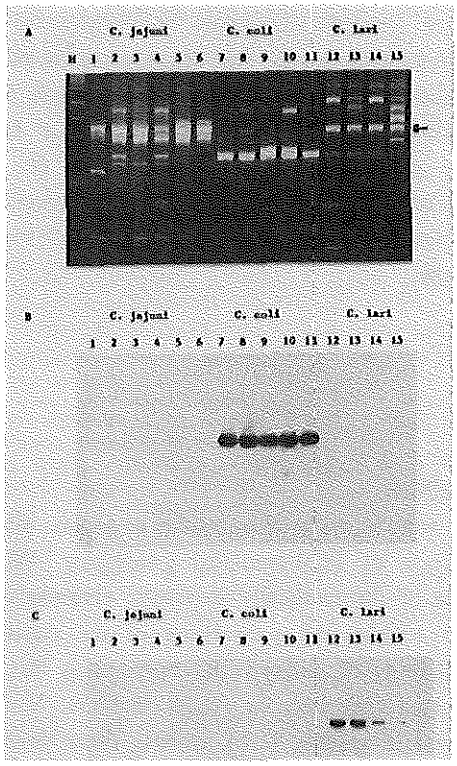


Figure 6 (A). Results of PCR amplification of DNA extracted from a selection of *C. jejuni, C. coli*, and *C. lari* strains. A combination of two primers (026 and ERIC2) was used. Lanes 1 to 15 show DNA banding patterns for *Campylobacter* strains *C. jejuni* subp. *jejuni* NCTC 11351, *C. jejuni* LIO 1, *C. jejuni* LIO 2, *C. jejuni* LIO 4, *C. jejuni* LIO 6, *C. jejuni* LIO 16, *C. jejuni*ATCC 33559, *C. coli* LIO 8, *C. coli* LIO 20, *C. coli* LIO 21, *C. coli* LIO 78, *C. lari* ATCC 35221, *C. lari* LIO 31, *C. lari* LIO 31, *C. lari* LIO 34, and *C. lari* LIO56, respectively; lane M, 1-kb DNA ladder. Arrows indicate the DNA fragment that were used as probes for Southern blot hybridizations; molecular size markers are identical to those displayed in Figures 4 and 5. **(B).** Southern blot hybridization with PCR products generated with the primer combination 1026 plus ERIC2 as a probe. The blot was hybridised with the 700-bp *C. coli* fragment. Lanes 1 to 15 contain the same strains as in panel A. **(C).** Southern blot hybridization with PCR products generated with primer combination 1026-ERIC2 as a probe. The blot was hybridised with the 1,100-bp *C. lari* fragment. Lanes 1 to 15 contain DNA from the same strains as in panel A. Reprinted from reference 35 with permission of the publisher.

REFERENCES

1. **Akopyanz, N., N.O. Bukanov, T.U. Westblom, S. Krurovich and D.E. Berg** (1992) DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Res. **20**: 5137-5142.
2. **Annas, J.D** (1992) Setting standards for the use of DNA-typing results in the courtroom - the state of the art. New Engl. J. Med. **326**: 1641-1644.
3. **Aufauvre-Brown, A., J. Cohen and D.W. Holden** (1992) Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. **30**: 2991-2993.
4. **Barany, F** (1991) The ligase chain reaction in a PCR world. PCR Meth. Appl. **5**: 5-16.
5. **Bassam, B.J., G. Caetano-Anollés and P.M. Gresshoff** (1992) DNA amplification fingerprinting of bacteria. Appl. Microbiol. Biotechnol. **38**: 70-76.
6. **Bej, A.K., M.H. Mahbubani and R.M. Atlas** (1991) Amplification of nucleic acids by polymerase chain reaction and other methods and their applications. Crit. Rev. Biochem. Mol. Biol. **26**: 301-334.
7. **Ben-Ezra, J., D.A. Johnson, J. Rossi, N. Cook and A. Wu** (1991) Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reactin. J. Histochem. Cytochem. **39**: 351-354.
8. **Birkenmeier, E.H., U. Schneider and S.J. Thurston** (1992) Fingerprinting genomes by use of PCR with primers that encode protein motifs or contain sequences that regulate gene expression. Mamm. Genom. **3**: 537-545.
9. **Bingen, E., E. Denamur, N. Lambert-Zechovsky, N. Brahimi, M. El-Lakany and J. Elion** (1992) Rapid genotyping shows the absence of cross-contamination in *Enterobacter cloacae* nosocomial infections. J. Hosp. Infect. **21**: 95-101.
10. **Bingen, E., C. Boissinot, P. Desjardins, H. Cave, N. Brahimi, N. Lambert, E. Denamur, P. Blot and J. Elion** (1993) Arbitrarily primed PCR provides rapid differentiation of *Proteus mirabilis* isolates from a pediatric hospital. J. Clin. Microbiol. **31**: 1055-1059.
11. **Boom, R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-van Dillen and J. van der Noordaa** (1990) Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28**: 495-503.
12. **Brousseau, R., A. Saint-Onge, G. Prefontaine, L. Masson and J. Cabana** (1993) Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. Appl. Environ. Microbiol. **59**: 114-119.
13. **Buck, G.E., L.C. O'Hara and J.T. Summersgill** (1992) Rapid, simple method for treating clinical specimens containing *Mycobacterium tuberculosis* to remove DNA for polymerase chain reaction. J. Clin. Microbiol. **30**: 1331-1334.
14. **Burstain, J.M., E. Grimprel, S.A. Lukehart, M.V. Norgard and J.D. Radolf** (1991) Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction. J. Clin. Microbiol. **29**: 62-69.
15. **Caetano-Annollés, G., B.J. Bassam and P.J. Gresshoff** (1992) Primer-template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides. Mol. Gen. Genet. **235**: 157-165.
16. **Cancilla, M.R., I.B. Powell, A.J. Hillier and B.E. Davidson** (1992) Rapid genomic fingerprinting of *Kluyveromyces lactis* strains by arbitrarily primed

polymerase chain reaction with 32P and fluorescent labels. Appl. Environ. Microbiol. **58**: 1772-1775.

17. **Cassol, S., J. Rudnik, T. Salas, M. Monpetit, R.T. Pon, C.T. Sy, S. Read, C. Major and M.V. O'Shaughnessy** (1992) Rapid DNA fingerprinting to control for specimen errors in HIV testing by the polymerase chain reaction. Mol. Cell. Probes **6**: 327-331.
18. **Chakraborty, R. and K.K. Kidd** (1991) The utility of DNA typing in forensic work. Science **254**: 1735-1739.
19. **Cohen, J** (1991) Clinical manifestations and management of aspergillosis in the compromised patient. p. 117-152. In: D.W. Warnock and M.D. Richardson (ed) Fungal infection in the compromised patient, 2nd ed., John Wiley, Chichester, U.K. (and other chapters therein).
20. **Colman, S.D., P.C. Hu and K.F. Bott** (1990) Prevalence of novel repeat sequences in and around the P1 operon in the genome of *Mycoplasma pneumoniae*. Gene **87**: 91-96.
21. **Correia, F.F., S. Inouye and M. Inouye** (1986) A 26 base-pair repetitive sequence specific for *Neisseria gonorrhoeae* and *Neisseria meningitidis* genomic DNA. J. Bacteriol. **167**: 1009-1015.
22. **Compton, J** (1991) Nucleic acid sequence based amplification. Nature **350**: 91-92.
23. **Crowhirst, R.A., B. Hawthorne, E.H.A. Rikkerink and M.D. Templeton** (1991) Differentiation of *Fusarium solani* f.sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Curr. Genet. **20**: 391-396.
24. **Czajka, J., N. Bsat, M. Piani, W. Russ, K. Sultana, M. Wiedmann, R. Whitaker and C.A. Batt** (1993) Differentiation of *Listeria monocytogenes* and *Listeria innocua* by 16S rRNA genes and intraspecies discrimination of *Listeria monocytogenes* strains by random amplified polymorphic DNA polymorphisms. Appl. Environ. Microbiol. **59**: 304-308.
25. **Dean, D., J. Schachter, C.R. Dawson and R.S. Stephens** (1992) Comparison of the major outer membrane protein variant sequence regions of B/Ba isolates: a molecular epidemiologic approach to *Chlamydia trachomatis* infections. J. Infect. Dis. **166**: 383-392.
26. **De Bruyn, F.J** (1992) Use of repetitive (REP and ERIC) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* and other soil bacteria. Appl. Environ. Microbiol. **58**: 3218-2187.
27. **Devlin, B., N. Risch and K. Roeder** (1993) Statistical evaluation of DNA fingerprinting: a critique of the NCR's report. Science **259**: 748-837.
28. **Eisenstein, B.I** (1990) New molecular techniques for microbial epidemiology and the diagnosis of infectious diseases. J. Infect. Dis. **161**: 595-602.
29. **Endtz, H., B. Giesendorf, A. van Belkum and W. Quint** (1993) PCR mediated typing of *Campylobacter jejuni* isolated from patients with recurrent infections. Microbiol. Res. **144**: 703-708.
30. **Espy, M.J., T.F. Smith and D.H. Persing** (1993) Dependence of PCR product inactivation protocols on amplicon length and sequence composition. J. Clin. Microbiol. **31**: 2361-2365.

31. **Fekete, A., J.A. Bantle, S.M. Halling and R.W. Stich** (1992) Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. J. Bacteriol. **174**: 7778-7783.
32. **Fujitani, S., T. Komano and S. Inouye** (1991) A unique repetitive DNA sequence in the *Myxococcus xanthus* genome. J. Bacteriol. **173**: 2125-2127.
33. **Guinée, P.A.M. and W.J. van Leeuwen** (1978) Phage typing of *Salmonella*. Methods Microbiol. **11**: 157-191.
34. **Giesendorf, B., A. van Belkum, A. Koeken, H. Stegeman, M. Henkens, J. van der Plas, B. Niesters and W. Quint** (1993) Development of species-specific DNA probes for *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* by PCR fingerprinting. J. Clin. Microbiol. **31**: 1541-1546.
35. **Giesendorf, B., H. Goossens, B. Niesters, A. van Belkum, H. Endtz, A. Koeken, H. Stegeman and W. Quint** (1993) PCR mediated DNA typing for epidemiological studies on *Campylobacter* species. J. Med. Microbiol. **40**: 141-147.
36. **Gilson, E., J.M. Clement, D. Brutlag and M. Hofnung** (1989) A family of dispersed repetitive, extragenic palindromic DNA sequences in *E. coli*. EMBO J. **3**: 1417-1421.
37. **Hagelberg, E., I.C. Gray and A.J. Jeffreys** (1991) Identification of the skeletal remains of a murder victim by DNA analysis. Nature **352**: 427-429.
38. **Hayes, L.J., R.L. Bailey, D.C. Mabey, I.N. Clarke, M.A. Pickett, P.J. Watt, M.E. Ward** (1992) Genotyping of *Chlamydia trachomatis* from a trachoma-endemic village in the Gambia by a nested polymerase chain reaction: identification of strain variants. J. Infect. Dis. **166**: 1173-1177.
39. **Hennessy, K., J.J. Iandolo and B.W. Fenwick** (1993) Serotype identification of *Actinobacillus pleuropneumoniae* by arbitrarily primed PCR. J. Clin. Microbiol. **31**: 1155-1159.
40. **Higgins, C.F., G.F.L. Ames, W.M. Barnes, J.M. Clement and M. Hofnung** (1982) A novel intercistronic regulatory element of prokaryotic operons. Nature **298**: 760-762.
41. **Hulton, C.S., C.F. Higgins and P.M. Sharp** (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other enterobacteria. Mol. Microbiol. **5**: 825-834.
42. **Janse, C.J., J. Ramesar and B. Mons** (1992) Chromosome translocation in *Plasmodium berghei*. Nucleic Acids Res. **20**: 581-586.
43. **Jayarao, B.M., B.J. Bassam, G. Caetano-Anolles, P.M. Gresshoff and S.P. Oliver** (1992) Subtyping of *Streptococcus uberis* by DNA amplification fingerprinting. J. Clin. Microbiol. **20**: 1347-1350.
44. **Jeffreys, A.J., V. Wilson and S.L. Thein** (1985) Hypervariable "mini-satellite" regions in human DNA. Nature **314**: 6773.
45. **Jeffreys, A.J., A. MacLeod, K. Tamaki, D.C. Neil and D.G. Monckton** (1991) Minisatellite repeat coding as a digital approach to DNA typing. Nature **354**: 204-209.
46. **Kersulyte, D., J.P. Woods, E.J. Keath, W.E. Goldman and D.E. Berg** (1992) Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. J. Bacteriol. **174**: 7075-7079.

47. **Kievits, T., B. van Gemen, D. van Strijp, R. Schukkink, M. Dircks, H. Adriaanse, L. Malek, R. Sooknanan and P. Lens** (1991) NASBA™ isothermal enzymatic in vitro nucleic acid amplification optimised for the diagnosis of HIV-1 infection. J. Vir. Meth. **35**: 273-286.
48. **Kramer, F.R. and M. Lizardi** (1989) Replicatable RNA reporters. Nature **339**:401-403.
49. **Kuypers, J.M., C.W. Critchlow, P.E. Gravitt, D.A. Vernon, J.B. Sayer, M.M. Manos and N.B. Kiviat** (1993) Comparison of dot filter hybridization, Southern transfer hybridization and polymerase chain reaction amplification for diagnosis of Human Papillomavirus infection. J. Clin. Microbiol. **31**: 1003-1006.
50. **Kwok, S. and R. Higuchi** (1989) Avoiding false positives with PCR. Nature **339**: 237-238.
51. **Lawyer, F.C., S. Stoffel, R.K. Saiki, K. Myambo, R. Drummond and D.H. Gelfand** (1989) Isolation, characterisation and expression in *E. coli* of the DNA polymerase gene from *Thermus aquaticus*. J. Biol. Chem. **264**: 6427-6437.
52. **Lehman, P.F., D. Lin and B.A. Lasker** (1992) Genotypic identification and characterisation of species and strains within the genus *Candida* by using random amplified polymorphic DNA. J. Clin. Microbiol. **30**: 3249-3254.
53. **Lennon, E., P.D. Gutman, H.L. Yao and K.W. Minton** (1991) A highly conserved repeated chromosomal sequence in the radioresistant bacterium *Deinococcus radiodurans* SARK. J. Bacteriol. **173**: 2137-2140.
54. **Levinson, G. and G.A. Gutman** (1987) Slipped strand mispairing: a major mechanism for DNA sequence evolution. Mol. Biol. Evol. **4**: 203-221.
55. **LiPuma, J.J., T.L. Stull, S.E. Dasen, K.A. Pidcock, D. Kaye and O.M. Korzeniowski** (1989) DNA polymorphisms among *Escherichia coli* isolated from bacteriuric women. J. Infect. Dis. **159**: 526-532.
56. **Lizardi, P.M., C.E. Guerra, H. Lomeli, I. Tussie-Lunie and F.R. Kramer** (1988) Exponential amplification of recombinant RNA hybridisation probes. Biotechnology **6**: 1197-1200.
57. **Loudon, K.W., J.P. Burni, A.P. Coke and R.C. Matthews** (1993) Application of PCR to fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA. J. Clin. Microbiol. **31**: 1117-1121.
58. **Lupski, J.R. and G.M. Weinstock** (1992) Short, interspersed repetitive DNA sequences in prokaryotic genomes. J. Bacteriol. **174**: 4525-4529.
59. **Manos, M.M., Y. Ting, D.K. Wright, A.J. Lewis, T.R. Broker and S.M. Walinsky** (1989) Use of PCR amplification for the detection of genital human papilloma viruses. Cancer Cells **7**: 209-214.
60. **Mazurier, S., A. Audurier, N. Marquet-Van der Meer, S. Notermans and K. Wernars** (1992) A comparative study of randomly amplified polymorphic DNA analysis and conventional phage typing for epidemiological studies of *Listeria monocytogenes* isolates. Res. Microbiol. **143**: 507-512.
61. **Mazurier, S. and K. Wernars** (1992) Typing of *Listeria* strains by random amplification of polymorphic DNA. Res. Microbiol. **143**: 499-505.
62. **Mazurier, S., A. van de Giessen, K. Heuvelman and K. Wernars** (1992) RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Lett. Appl. Microbiol. **14**: 260-262.

63. **Menard, C., R. Brousseau and C. Muton** (1992) Application of polymerase chain reaction with arbitrary primer (AP-PCR) to strain identification of *Porphyromonas (Bacteroides) gingivalis*. FEMS Microbiol. Lett. **74**: 163-168.
64. **Meyers, L.E., S.V.P.S. Silva, J.D. Procunier and P.B. Little** (1993) Genomic fingerprinting of *Haemophilus somnus* isolates by using a random-amplified polymorphic DNA assay. J. Clin. Microbiol. **31**: 512-517.
65. **Nastasi, A., S. Pignato, C. Mammina and G. Giammanco** (1993) rRNA gene restriction patterns and biotypes of *Shigella sonnei*. Epidemiol. Infect. **110**: 23-30.
66. **Niesters, H.G.M., W. Goessens, J.F.G.M. Meis and W.G.V. Quint** (1993) Rapid PCR based identification assays for *Candida* species. J. Clin. Microbiol. **31**: 903-908.
67. **Pääbo, S., R.G. Higuchi and A.C. Wilson** (1989) Ancient DNA and the polymerase chain reaction. J. Biol. Chem. **264**: 9709-9712.
68. **Patton, C.M., I.K. Wachsmuth, G.M. Evins, J.A. Kiehlbauch, B.D. Plikaytis, N. Troyo, L. Tompkins and H. Lior** (1991) Evaluation of 10 methods to distinguish epidemic-associated *Campylobacter* strains. J. Clin. Microbiol. **29**: 680-688.
69. **Persing, P.H. and M.L. Landry** (1989) In vitro amplification techniques for the detection of nucleic acids: new tools for the diagnostic laboratory. Yale J. Biol. Med. **62**: 159-171.
70. **Peter, J.B** (1991) The polymerase chain reaction: amplifying our options. Rev. Infect. Dis. **13**: 166-171.
71. **Peterson, W.L** (1991) *Helicobacter pylori* and peptic ulcer disease. New Engl. J. Med. **324**: 1043-1048.
72. **Pfaller, M.A** (1991) Typing methods for epidemiologic investigations. In: Manual of Clinical Microbiology, 5th ed., Ed. A. Balows et al., Washington DC, ASM, pp. 171-182.
73. **Pfaller, M.A** (1992) Epidemiological typing methods for mycoses. Clin. Infect. Dis. **14**: S4-S10.
74. **Puchhammer-Stöckl E., F.X. Heinz, M. Kundi, T. Popow-Kraupp, G. Grimm, M.M. Millner and C. Kunz** (1993) Evaluation of the polymerase chain reaction for diagnosis of Herpes Simplex Virus encephalitis. J. Clin. Microbiol. **31**: 146-148.
75. **Ralph, D., M. McClelland, J. Welsh, G. Baranton and P. Perolat** (1993) *Leptospira* species categorised by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. J. Bacteriol. **175**: 973-981.
76. **Riley, D.E., M. Samadpour and J.N. Krieger** (1991) Detection of variable DNA repeats in diverse eukaryotic microorganisms by a single set of PCR primers. J. Clin. Microbiol. **29**: 2746-2751.
77. **Ross, B.C. and B. Dwyer** (1993) Rapid, simple method for typing isolates of *Mycobacterium tuberculosis* by using the PCR. J. Clin. Microbiol. **31**: 329-334.
78. **Rys, P.N. and D.H. Persing** (1993) Preventing false positives: quantitative evaluation of three protocols for inactivation of PCR amplification products. J. Clin. Microbiol. **31**: 2356-2360.

79. **Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Hory, K.B. Mullis and H.A. Erlich** (1988) Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**: 487-491.
80. **Sarafis, K. and J. Isaac-Renton** (1993) Pulsed-field gel electrophoresis as a method of biotyping of *Giardia duodenalis*. Am. J. Trop. Med. Hyg. **48**: 134-144.
81. **Saurnier, P., C. Bourneux, G. Prévost and A. Andremont** (1993) RAPD assay is less discriminant than pulsed-field gel electrophoresis for typing strains of methicillin-resistant *S. aureus*. J. Clin. Microbiol. **31**: 982-985.
82. **Seal, S.E., L.A. Jackson and M.J. Daniels** (1992) Use of tRNA consensus primers to indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction amplification. Appl. Environ. Microbiol. **58**: 3759-3761.
83. **Sharples, G.J. and R.G. Lloyd** (1990) A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. Nucleic Acids Res. **18**: 6503-6508.
84. **Sheffield, V.C., D.R. Cox, L.R. Lerman and R.M. Meyers** (1989) Attachment of a 40 basepair G+C rich sequence (GC Clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single base changes. Proc. Natl. Acad. Sci. USA **86**: 232-236.
85. **Sibley, L.D. and J.C. Boothroyd** (1992) Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature **359**: 82-85.
86. **Skakni, L., A. Sardet, J. Just, J. Landman-Parker, J. Costil, N. Moniot-Ville, F. Bricout and A. Garbarg-Chenon** (1992) Detection of *Mycoplasma pneumoniae* in clinical samples from pediatric patients by polymerase chain reaction. J. Clin. Microbiol. **30**: 2638-2643.
87. **Smith, C.L. and C.R. Cantor** (1987) Purification, specific fragmentation and separation of large DNA molecules. Methods Enzymol. **155**: 449-467.
88. **Smith, M.L., J.N. Bruk and J.B. Anderson** (1992) The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. Nature **356**: 428-431.
89. **Snijders, P.J.F., A. van den Brule, H. Schrijnemakers, G. Snow, C. Meyer and J.M.M. Walboomers** (1990) The use of general primers in the PCR permits the detection of a broad spectrum of human papillomavirus infections. J. Gen. Virol. **72**: 2781-2786.
90. **Struelens, M.J., N. Maes, F. Rost, A. Deplano, F. Jacobs, C. Liesnard, N. Bornstein, F. Grimont, S. Lauwers, M.P. McIntyre and E. Serruys** (1992) Genotypic and phenotypic methods for the investigation of a nosocomial *Legionella pneumophila* outbreak and efficacy of control measures. J. Infect. Dis. **166**: 22-30.
91. **Struelens, M., R. Bax, A. Dephano, W. Quint and A. van Belkum** (1993) Concordant clonal delineation of methicillin-resistant *S. aureus* by macrorestriction analysis and PCR genome fingerprinting. J. Clin. Microbiol. **31**, 1964-1970.
92. **Struelens, M., E. Carlier, N. Maes, E. Serruys, W. Quint and A. van Belkum** (1993) Nosocomial colonisation and infection with multiresistant *Acinetobacter baumannii*: outbreak delineation using DNA macrorestriction analysis and PCR fingerprinting. J. Hosp. Inf. **15**: 15-32.
93. **Stull, T.C., J.J. LiPuma and T.D. Edlind** (1988) A broad spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. J. Infect. Dis. **157**: 280-86.

94. **Subramanian, P.S., J. Versalovic, E.R.B. McCabe and J.R. Lupski** (1992) Rapid mapping of *E. coli* Tn5 insertion mutations by REP-Tn5 PCR. PCR Meth. Appl. **1**: 187-194.
95. **Tibayrenc, M., K. Neubauer, C. Barnabé, F. Guerrini, D. Skarecky and F.J. Ayala** (1993) Genetic characterisation of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. Proc. Natl. Acad. Sci. USA **90**: 1335-1339.
96. **Uijthof, J., A. de Cock, S. de Hoog, W. Quint and A. van Belkum** (1994) PCR mediated genotyping of *Hortaea werneckii*, causative agent of tinea nigra. Mycology, **37**: 307-312.
97. **Upcroft, P., R. Mitchell and P.F.L. Boreham** (1990) DNA fingerprinting of the intestinal parasite *Giardia duodenalis* with the M13 phage genome. Int. J. Parasitol. **20**: 319-323.
98. **Van Belkum, A., G. Trommelen and A. Uitterlinden** (1991) Minisatellite-like repeat sequences in the genome of rodent malaria parasites. Acta Leidensia **60**: 83-91.
99. **Van Belkum, A., J. Ramesar, G. Trommelen and A.G. Uitterlinden** (1992) Mini- and microsatellites in the genome of malaria parasites. Gene **118**: 81-86.
100. **Van Belkum, A., W. Homan, Limper L. and W.G.V. Quint** (1993) Genotyping isolates and clones of *Giardia duodenalis* by PCR: implications for the detection of clonality in protozoan parasites. Mol. Biochem. Parasitol. **61**: 68-79.
101. **Van Belkum, A., R. Bax, P. Peerbooms, W. Goessens, N. van Leeuwen and W.G.V. Quint** (1993) Comparison of phage typing and DNA fingerprinting by PCR for discrimination of methicillin-resistant *S. aureus* strains. J. Clin. Microbiol. **31**; 798-803.
102. **Van Belkum, A., R. Bax, P. van Straaten, W. Quint and E. Veringa** (1994) Monitoring nosocomial *S. aureus* infections with PCR fingerprinting. J. Bacteriol. Meth., **20**: 235-247.
103. **Van Belkum A., J.F. de Jonckheere and W.G.V. Quint** (1992) Genotyping of *Naegleria* spp. and *Naegleria fowleri* isolates by interrepeat PCR. J. Clin. Microbiol. **30**: 2595-2598.
104. **Van Belkum, A., M. Struelens and W. Quint** (1993) Typing of *Legionella pneumophila* strains by polymerase chain reaction-mediated DNA fingerprinting. J. Clin. Microbiol. **31**: 2198-2200.
105. **Van Belkum A., W.G.V. Quint, B. de Pauw, W. Melchers and J. Meis** (1993) Typing of *Aspergillus* species and *Aspergillus fumigatus* isolates by interrepeat polymerase chain reaction. J. Clin. Microbiol. **31**: 2502-2505.
106. **Van Doorn, L.J** (1994) Molecular characterisation of the hepatitis C virus genome. Ph.D. Thesis, Erasmus University Rotterdam, The Netherlands.
107. **Versalovic, J., T. Koeuth and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19**: 6823-6831.
108. **Vogt, P** (1990) Potential genetic functions of tandem repeated DNA sequence blocks in the human genome are based on a highly conserved "chromatin folding code". Hum. Genet. **84**: 301-336.

109. **Wakefield, A.E., L. Guiver, R.F. Miller and J.M. Hopkin** (1991) DNA amplification on induced sputum samples for diagnosis of *Pneumocystis carinii* pneumonia. Lancet **337**: 1378-1379.
110. **Welsh, J. and M. McClelland** (1991) Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combination of primers. Nucleic Acids Res. **19**: 5275-5279.
111. **Welsh, J., C. Petersen and M. McClelland** (1991) Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. Nucleic Acids Res. **19**: 303-306.
112. **Welsh, J., K. Chada, S.S. Dalal, R. Cheng, D. Ralph and M. McClelland** (1992) Arbitrary primed PCR fingerprinting of RNA. Nucleic Acids Res. **20**: 4965-4970.
113. **Welsh, J., C. Pretzman, D. Postic, I. Saint-Girons, G. Baranton and M. McClelland** (1992) Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* to three distinct phyletic groups. Int. J. Syst. Bacteriol. **42**: 370-377.
114. **Wenzel, R. and R. Herrmann** (1988) Repetitive DNA sequences in *Mycoplasma pneumoniae*. Nucleic Acids Res. **16**: 8337-8350.
115. **White, T.J., N. Arnheim and H.A. Erlich** (1989) The polymerase chain reaction. Trends in Genetics **5**: 185-189.
116. **Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey** (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18**: 6531-6535.
117. **Wilske, B., A.G. Brbour, S. Bergstrom, N. Burman, B.I. Restrepo, P.A. Rosa, T. Schwan, E. Soutschek and R. Wallich** (1992) Antigenic variation and strain heterogeneity in *Borrelia spp.*. Res. Microbiol. **143**: 583-596.
118. **Wilson, M.A., R.B. Rinker and L.J. Hoffman** (1992) Comparison of DNA fingerprints and somatic serotypes of serogroup B and E *Pasteurella multicida* isolates. J. Clin. Microbiol. **30**: 1518-1524.
119. **Woods, J.P., D. Kersulyte, W.E. Goldman and D.E. Berg** (1993) Fast DNA isolation from *Histoplasma capsulatum*: Methodology for arbitrary primer polymerase chain reaction-based epidemiological and clinical studies. J. Clin. Microbiol. **31**: 463-464.
120. **Woods, C.R., J. Versalovic, T. Koeuth and J.R. Lupski** (1992) Analysis of relationships among isolates of *Citrobacter diversus* by using DNA fingerprints generated by repetitive sequence-based primers in the polymerase chain reaction. J. Clin. Microbiol. **30**: 2921-2929.
121. **Woods, C.R., J. Versalovic, T. Koeuth and J.R. Lupski** (1993) Whole cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. J. Clin. Microbiol. 31, in press.
122. **Ziegle, J.S., Y. Su, K.P. Corcoran, L. Nie, P.E. Maurand, L.B. Hoff, L.J. McBride, M.N. Kronick and S.R. Diehl** (1992) Application of automated DNA sizing technology for genotyping microsatellite loci. Genomics **14**: 1026-1031.

Adapted from: J. Clin. Microbiol. 1992;30:2595-2598.

CHAPTER III

GENOTYPING *NAEGLERIA* SPP AND *NAEGLERIA FOWLERI* ISOLATES BY INTERREPEAT PCR

Alex van Belkum, Johan De Jonckheere[1] and Wim G.V. Quint[2]

[1]Institute for Hygiene and Epidemiology, Juliette Wytsmanstraat 14, 1050 Brussels, Belgium. [2]Diagnostic Centre SSDZ, Department of Molecular Biology, Reinier de Graafweg 7, P.O. Box 5010, 2600 GA Delft, The Netherlands.

SUMMARY

All six *Naegleria* species recognized to date were studied by interrepeat polymerase chain reaction (IR PCR). Priming at repeat sequences which are known to be variable among eukaryotes, yielded electrophoretic DNA banding patterns that were specific for any single species. With a single PCR and simple gel electrophoresis, species determination could be performed in less than one day. Unambiguous discrimination between the pathogen *N. fowleri* and non-pathogenic *Naegleria* species appeared to be possible. Analysis of DNA obtained from 20 separate isolates of *N. fowleri* revealed that geographic variation of the genetic fingerprints rarely occurs. All but three isolates of *N. fowleri* which were investigated showed identical banding patterns: a limited number of additional bands was detected for two isolates from New Zealand and one from Australia, independent of the PCR primers used. These data corroborate previous findings on the genetic stability of pathogenic *N. fowleri*.

INTRODUCTION

The genus *Naegleria* consists of free-living amoeboflagellates, among which the species *N. fowleri* is implicated as the causative agent of primary amoebic meningoencephalitis (10). Since this type of encephalitis is lethal for most patients, highly sensitive and specific detection strategies are required for successful recognition of the pathogen at a stage of infection at which clinical intervention is still feasible (16). Prevention of infections by screening the environment for potential habitats also requires the availability of sensitive and specific tests. For this aim, various tests for the detection and identification of restriction fragment length polymorphisms (RFLPs) have been reported for *Naegleria* spp as well as for *Acanthamoeba* spp, another amoeba that produces meningitis (1, 2, 8, 11, 13, 21). Most of these polymorphisms are detected within concatameric ribosomal operons or the mitochondrial genome. RFLP studies require relatively large amounts of DNA and involve laborious electrophoresis, blotting, and hybridisation techniques. Alternatively, detection of the genomic DNA of *Naegleria* spp. can be performed with increased simplicity and sensitivity by the polymerase chain reaction (PCR) (7). Recently, a repetitive DNA sequence from the mitochondrial DNA of *N. fowleri*, which probably codes for the ATPase 6 subunit, was sequenced and a species-specific assay was

Table 1 Collection of *Naegleria* strains included in this study.

Naegleria species	reference code	origin	human isolate	year of isolation
N. fowleri	Lovell	USA	+	1974
	WM	USA	+	1969
	LEE-1 (ATCC 30894)	USA	+	1968
	124	USA	-	1984
	Enterprise	USA	-	1976
	HB-1	USA	+	1966
	SW1	USA	-	1976
	TY	USA	+	1969
	Mst	New Zealand	+	1974
	NHI	New Zealand	+	1974
	PA34 (ATCC 30468)	Australia	-	1972
	Northcott (ATCC 30462)	Australia	+	1971
	PAa	Australia	-	1972
	ORAM (ATCC 30463)	Australia	+	1971
	J26/50/42C	Japan	-	1990
	J16/I/42E	Japan	-	1990
	M4E	France	-	1979
	MCM	England	+	1978
	NA3	India	-	1972
	T37(50) F44	Belgium	-	1981
	KUL (ATCC 30808)	Belgium	+	1973
N. lovaniensis	Aq/9/1/45D	Belgium	-	1976
N. gruberi	CCAP 1518/1e (ATCC 30876)	USA	-	1964
N. jadini	0.400 (ATCC 30900)	Belgium	-	1972
N. australiensis australiensis	PP397 (ATCC 30958)	Australia	-	1973
N. australiensis italica	AB-T-F3	Italy	-	1982
N. andersoni andersoni	PPMFB-6	Australia	-	1972
N. andersoni jamiesoni	T56E	Singapore	-	1981

developed for *N. fowleri* (12). By altering the hybridization stringency of the PCR assay, other, non-pathogenic *Naegleria* spp. could also be detected, but no identification of species could be performed.

In this report, a PCR procedure that enables the detection of variable DNA sequences in lower eukaryotes without prior knowledge of genomic DNA sequences is described. By using PCR-priming oligonucleotides targeted at sequences known to be polymorphic in higher and lower eukaryotes (9), species-specific fingerprints were obtained. A number of primers suitable for identification of all separate *Naegleria* species were also used in combination with DNA isolates from isolates of *N. fowleri* obtained from throughout the world.

MATERIALS AND METHODS

Six *Naegleria* spp and several isolates and subspecies were grown and harvested by established procedures. Cultivation was performed by using Serum-Casein-Glucose-Yeast extract medium (SCCYEM) containing calf serum (3). Amoebic DNA was isolated by phenol extractions as described previously (4). Table 1 lists all species and strains examined in this study. For PCR analysis, 10 primers were initially evaluated with respect to their applicability in genotyping studies. All oligonucleotides used in the study were prepared on an ABI 386 DNA synthesizer using fosforamidite technology. The PCR conditions, which were optimized using various yeast DNAs as template (results not shown), were identical in all experiments. PCR was performed in 100 μl volumes containing 10 mM Tris.HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM desoxynucleotide triphosphate, 100 pmol of a single primer oligonucleotide and between 0.1 and 1 μg template DNA. For each reaction 0.25 units of Taq DNA polymerase (Super Taq, Sphaero Q, Leiden, The Netherlands) was included, and 40 reaction cycles (1 min 94°C, 2 min 52°C or 2 min 42°C, 3 min 74°C) were performed in a Biomed thermocycler (type 68). PCR products were separated on 3% agarose gels and stained with ethidium bromide.

RESULTS

A number of PCR primers were synthesized with nucleotide sequences deduced from variable repeats that occur in the human genome (22). The various interrepeat primers reacted differentially with the *Naegleria* DNA preparations. Three oligonucleotides consisting of di- or tri-nucleotide repeats did not give rise to the PCR-mediated synthesis of a single DNA fragment. In the case of a trinucleotide and a tetranucleotide repeat, only DNA smears were seen. The mere fact that DNA-synthesis took place with these primers indicates that optimization of the reaction parameters could lead to the generation of adequate DNA fingerprints. An oligonucleotide consisting of four GACT repeats reacted with *N. lovaniensis* and *N. australiensis subsp. australiensis* only: single DNA fragments (approximately 4000 and 1000 basepairs, respectively) were synthesized (data not shown). A primer containing 3 repeats of the human telomeric consensus sequence (TTAGGG) gave rise to polymorphic DNA fingerprints, albeit with a very low

DNA-synthesizing efficiency. By using this primer, *N. jadini* could not be discriminated from *N. australiensis subsp. italica* because identical DNA banding patterns were found. However, several other telomer-specific consensus sequences have been described (24). Application of another telomer specific primer ($(TGGGTGTG)_3$, primer 9) led to the generation of complex fingerprints for all *Naegleria* spp. A pentamer of the simple sequence motif GACA (primer code 0) or a heptamer of the simple sequence motif GGA

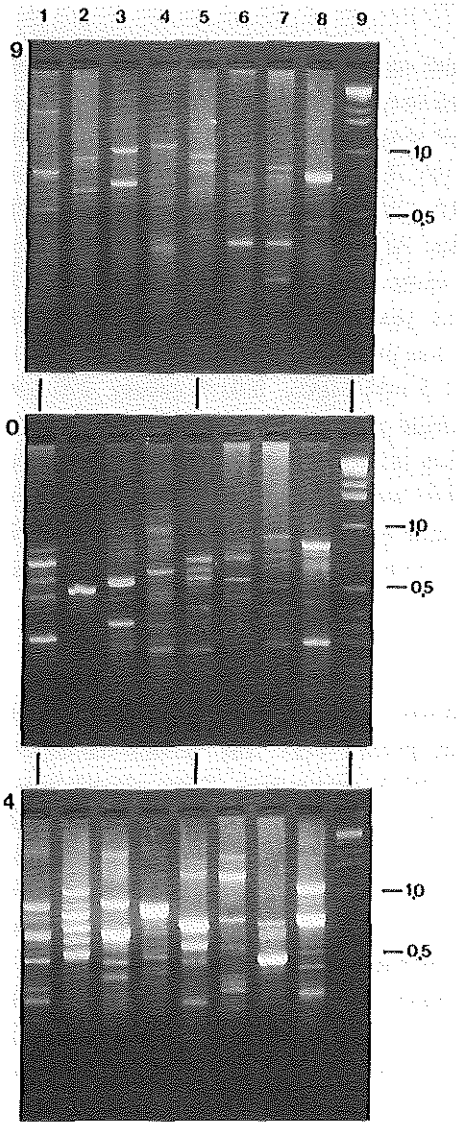


Figure 1 Interrepeat PCR with oligonucleotides specific for polymorphic regions in the genomes of *Naegleria* spp. Oligonucleotides are specific for consensus telomeric sequences (primer 9, $(TGGGTGTG)_3$) and two types of simple sequence motifs (primer 0, $(GACA)_5$ and primer 4, $(GGA)_7$). Shown are three electropherograms displaying the results of interrepeat PCR at an annealing temperature of 42°C on DNA from various *Naegleria* spp. (A. primer 9, B. primer 0, C. primer 4). From left to right (lanes 1 to 8) DNA from the following organisms was used as template: *N. andersoni subsp. andersoni*, *N. fowleri* (KUL), *N. andersoni subsp. jamiesoni*, *N. australiensis subsp. italica*, *N. lovaniensis*, *N. gruberi*, *N. australiensis subsp. australiensis*, *N. jadini*. Lane 9 displays molecular weight markers, and their length is indicated in kilobasepairs on the right. Species characteristics are described in Table 1.

(primer code 4) also gave rise to clear, polymorphic DNA banding patterns (Figure 1). For the GACA repeats, it has been demonstrated that even in clonal populations of two fish species, genetic variability could be detected (17). All three primers mentioned above allowed the discrimination of any species from another, enabling species determination by a single PCR. An increase in the annealing temperature from 42°C to 52°C lead to a small change in the DNA profile.

In order to analyze the genetic stability of the *Naegleria* genome and the reproducibility of the interrepeat PCR, 20 isolates of *N. fowleri* with different geographic origin were studied. Figure 2 shows the result of interrepeat PCR with oligonucleotide primers 9 and 4. For all but three isolates, identical patterns were generated, indicating the intraspecies stability of the loci targeted by the interrepeat PCR. Additional bands were found only for 2 isolates from New Zealand and one isolate from Australia when either of the two primer species was used. This was not the case when primer 0 was applied: in that case, all patterns were identical.

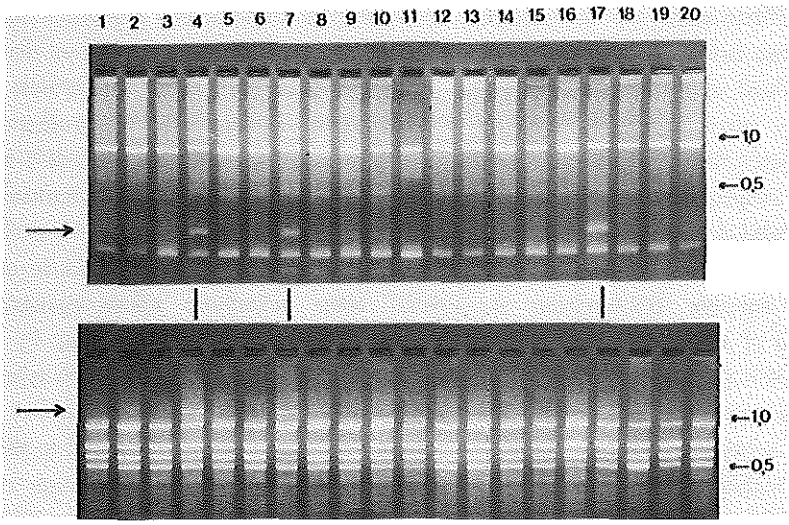


Figure 2 Comparison of interrepeat PCR products synthesized on DNA isolated from *Naegleria fowleri* from throughout the world. Primers 9 (A) and 4 (B) were used. From left to right (1-20) the following isolates were studied: TY, T37 (50) F44, NA3, NHI, MCM, SW1, Mst, Enterprise, M4E, HB-1, J16/1/42E, ORAM (ATCC30463), 124, LEE-1 (ATCC 30894), WM, J26/50/42C, PAa, Northcott (ATCC 30462), PA34 (ATCC 30468) and Lovell. On the right molecular weight markers are indicated in kilobases, the arrows on the left point to bands that were visible in the isolates of the New Zealand subtype (lanes with New Zealand subtype DNA are connected by a vertical line). By using primer 0, a single band was observed in all isolates, including those of the New Zealand subtype (data not shown). Note that the primer 4 pattern looks different from the corresponding lanes in Figure 1. This was due to an increased annealing temperature.

DISCUSSION

Since little is known about the distribution of repetitive DNAs throughout the genome of *Naegleria* (12) and about the role that these sequences can play in species identification, a genetic analysis of several species by using PCR primers aimed at telomeric and simple repeat sequences was performed. It was demonstrated that *Naegleria* isolates can be identified to species and subspecies levels by a single PCR (Figure 1). Characteristic DNA banding patterns (so-called fingerprints) can be obtained by the application of oligonucleotide primers specific for eukaryotic DNA regions which are documented to be variable. The sequence motifs used occur in eukaryotic DNA as elements that consist of short, usually GC-rich sequence units iterated in tandem and, as such, form arrays of between 0.1 and 20 kilobasepairs in length (9). These sequences are also used in the genetic analysis of humans involved in, for instance, paternity disputes or forensic cases (9, 14). Allelic variation as observed among human individuals is apparently absent between isolates of the same *Naegleria* sp. This is in line with observations on the occurrence of repetitive sequences in lower eukaryotes. In *Caenorhabditis elegans* and several *Plasmodium* spp for instance, monomorphic minisatellite sequences have been found when isolates of a single species were studied (18, 20). It is interesting that various well defined subspecies of *Naegleria* (e.g. *N. andersoni subsp. andersoni* and *N. andersoni subsp. jamiesoni* or *N. australiensis subsp. italica* and *N. australiensis subsp. australiensis*) display completely different fingerprints (Figure 1, lanes 1 and 3 and lanes 4 and 7, respectively). This implies that these subspecies can also be discriminated on the basis of well-defined genetic characteristics. It has been documented that the performance of PCR specific for a DNA repeat initially isolated from *Trichomonas vaginalis* can detect similar, variable DNA-repeats in several other eukaryotic micro-organisms (15). Detection of DNA polymorphisms can also be done in a more general way by arbitrary primed PCR (23). By using short oligonucleotides, complex genomes can be typed easily. The disadvantage of the use of short primers is the relatively large number of PCR products synthesized. Furthermore, the correct choice of a primer is crucial to the success of the analysis. The procedure described here circumvents the generation of highly complex fingerprints. A limited number of hybridisation sites is selected, but on the other hand, the degree of variability is retained by aiming at known, highly variable regions in the DNA. The complexity of the banding pattern can be modulated by altering the annealing temperature. An increase in the annealing temperature leads to simplification of the patterns (compare Figure 1 lane 2, and Figure 2), probably by avoiding illegitimate PCR initiation on primer-template combinations that are not fully homologous.

The procedure described here can be used for the discrimination of the pathogenic amoeba *N. fowleri* from the other non-pathogenic ones. Different isolates appear to contain monomorphic repeat loci, although some isolates (one from Australia and two from New Zealand) display limited variabilities when compared to isolates with a different geographic origin. Restriction fragment length polymorphism of whole-cell DNA

from *N. fowleri* showed one type of this species in Europe, while in Australia a different type was detected together with a different subtype that was found in New Zealand (4, 5). Isolates with both the European and Australian types, together with isolates with intermediate patterns, are present on the North-American continent. Strains of *N. fowleri* isolated recently in Japan (6) were different from, but most had patterns related to the Australian pattern. The interrepeat PCR with primers reported here could only differentiate the New Zealand and Australian subtypes. Although our results show that the geographic origin of a *N. fowleri* isolate cannot be determined with the primers used in the interepeat PCR, the method described here is a highly specific and rapid way of identifying the pathogenic species. The strains of *N. fowleri* used in this study had been in culture for various periods of time, which is known to change their virulence (3). The interrepeat PCR banding pattern does not seem to differ according to the degree of pathogenicity of the isolate. Also, DNA from other lower eukaryotic organisms can be subjected to this DNA typing approach. The general method can be of use in the analysis of epidemiological spreading of microbes or for the direct isolation of (sub)species-specific DNA probes. Forthcoming research will focus on the precise identity of the amplified *Naegleria* DNA, whereas studies on the applicability of this type of research in clinical diagnostics will be initiated.

REFERENCES

1. **Bogler, S.A., C.D. Zarley, L.L. Buranek, P.A. Fuerst and T.J. Byers** (1983) Interstrain mitochondrial DNA polymorphism detected in *Acanthamoeba* by restriction endonuclease analysis. Mol. Biochem. Parasitol. **8**: 145-163.
2. **Costas, M., S.W. Edwards, D. Lloyd, A. Griffiths and G. Turner** (1983) Restriction enzyme analysis of mitochondrial DNA of members of the genus *Acanthamoeba* as an aid in taxonomy. FEMS Microbiol. Lett. **7**: 231-234.
3. **De Jonckheere, J.F** (1979) Differences in virulence of *Naegleria fowleri*. Pathol. Biol. **27**: 453-458.
4. **De Jonckheere, J.F** (1987) Characterisation of *Naegleria* species by restriction endonuclease digestion of whole cell DNA. Mol. Biochem. Parasitol. **24**: 55-66.
5. **De Jonckheere J.F** (1988) Geographic origin and spread of pathogenic *Naegleria fowleri* deduced from restriction enzyme patterns of repeated DNA. Biosystems **21**: 269-275.
6. **De Jonckheere J.F., K. Yagita, T. Kuroki and T. Endo** (1991) First isolation of pathogenic *Naegleria fowleri* from Japan. Jpn. J. Parasitol. **40**: 352-357.
7. **Erlich, H.A., D. Gelfland and J.J. Sninsky** (1991) Recent advances in the polymerase chain reaction. Science **252**: 1643-1651.
8. **Gunderson, J.H. and M.L. Sogin** (1986) Length variation in eukaryotic rRNAs: small subunit rRNAs from the protists *Acanthamoeba castellanii* and *Euglena gracilis*. Gene **44**: 63-70.
9. **Jeffreys, A.J., V. Wilson and S.L. Thein** (1985) Hypervariable minisatellite regions in human DNA. Nature **314**: 67-73.
10. **Martinez, A.J.** (1985) Free-living amebas: natural history, diagnostis, pathology, and treatment of disease, CRC Press Inc., Boca Raton, Fla.

11. **McLaughlin, G.L., F.H. Brandt and G.S. Visvesvara** (1988) Restriction fragment length polymorphisms of the DNA of selected *Naegleria* and *Acanthamoeba amoeba*. J. Clin. Microbiol. **26**: 1655-1658.
12. **McLaughlin G.L., M.H. Vodkin and H.W. Huizinga** (1991) Amplification of repetitive DNA for the specific detection of *Naegleria fowleri*. J. clin. Microbiol. **29**: 227-230.
13. **Milligan, S.M. and R.N. Band** (1988) Restriction endonuclease analysis of mitochondrial DNA as an aid in the taxonomy of *Naegleria* and *Vahlkampfia*. J. Protozool. **35**: 198-204.
14. **Nakamura, Y., M. Leppert, P. O'Connell, R. Wolff, T. Hohn, M. Ailver, C. Martin, E. Fujuimoto, M. Hoff, E. Kumlin and R. White** (1987) Variable number of tandem repeats (VNTR) markers for human gene mapping. Science **235**: 1616-1622.
15. **Riley D.E., M. Samadpour and J.N. Krieger** (1991) Detection of variable DNA repeats in diverse eukaryotic microorganisms by a single set of polymerase chain reaction primers. J. Clin. Microbiol. **29**: 2746-2751.
16. **Seidel, J.S., P. Harmatz, G.S.Visvesvara, A. Cohen, J. Edwards and J. Turner** (1982) Successful treatment of primary amoebic meningoencephalitis. N. Engl. J. Med. **306**: 346-348.
17. **Turner, B.J., J.F. Elder, T.F. Laughlin and W.P. Davies** (1990) Genetic variation in clonal vertebrates detected by simple-sequence DNA fingerprinting. Proc. Natl. Acad. Sci. USA **87**: 5653-5657.
18. **Uitterlinden A.G., P.E. Slagboom, T.E. Johnson and J. Vijg** (1989) The *Caenorhabditis elegans* genome contains monomorphic minisatellites and simple sequences. Nucleic Acids Res. **17**: 9527-9530.
19. **Upcroft, P., R. Mitchell and P.F.L. Boreham** (1990) DNA fingerprinting of the intestinal parasite *Giardia duodenalis* with the M13 phage genome. Int. J. Parasitol. **20**: 319-323.
20. **Van Belkum A., G. Trommelen and and A. Uitterlinden** (1991) Minisatellite-like repeat sequences in the genome of rodent malaria parasites. Acta Leidensia **60**: 83-91.
21. **Vodkin, M.H., D.K. Howe, G.S. Visvesvara and G.L. McLaughlin** (1992) Identification of *Acanthamoeba* at the generic and specific levels using the polymerase chain reaction. J. Protozool. **39**: 378-385.
22. **Vogt, P** (1990) Potential genetic functions of tandem repeated sequence blocks in the human genome are based on a highly conserved chromatin folding code. Hum. Genet. **4**: 301-336.
23. **Welsh J., C. Petersen and M. McClelland** (1991) Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. Nucleic Acids Res. **19**: 303-306.
24. **Zakian, V.A** (1989) Structure and function of telomers. Ann. Rev. Genet. **23**: 579-604.

Adapted from: Mol. Biol. Parasitol. 1993;61:69-78.

CHAPTER IV

GENOTYPING ISOLATES AND CLONES OF *GIARDIA DUODENALIS* BY POLYMERASE CHAIN REACTION: IMPLICATIONS FOR THE DETECTION OF GENETIC VARIATION AMONG PROTOZOAN PARASITE SPECIES

Alex van Belkum, Wieger Homan[1], Louis Limper[1] and Wim G.V. Quint[2]

[1]National Institute of Public Health and Environmental Protection, P.O. Box 1, 8720 BA Bilthoven, The Netherlands. [2]Diagnostic Center SSDZ, Department of Molecular Biology, P.O. Box 5010, 2600 GA Delft, The Netherlands.

SUMMARY

Detection of genetic variation among microorganisms can be done by DNA fingerprinting using the polymerase chain reaction (PCR). Application of primers directed to polymorphic DNA leads to the amplification of DNA fragments which differ in length when different species or isolates of a single species are compared. It has been demonstrated that PCR primers resembling eukaryotic repeat motifs enable the straightforward genetic differentiation of *Giardia duodenalis* isolates. Depending on the repeat motif, genetic variation between cloned *G. duodenalis* lines could also be detected. DNA polymorphisms could also be detected by random amplification of polymorphic DNA (RAPD) analyses. When the results obtained for *G. duodenalis* are compared to those found for another protozoan parasite, *Naegleria fowleri*, clear differences are encountered. In contrast to the findings for *G. duodenalis*, the repeat motif primers did not allow the discrimination of *N. fowleri* isolates. Apparently, as determined by this PCR-mediated genotyping, genetic variation occurs in *G. duodenalis* with increased frequency at the isolate level as compared to *N. fowleri*. The possible implications of this observation for clonality or the definition of a species in protozoan parasites will be discussed.

INTRODUCTION

Protozoan parasites display a strong tendency towards genetic variation (22). The programmed alternation of surface antigens in *Trypanosoma* species for instance provides a sophisticated example of how a parasite can escape from the host's immune response by genetic modification (19). Also, more randomly occurring chromosomal rearrangements have been described in many parasites (7). Information on the genomic composition of parasite isolates is important for establishing phylogenetic relationships or for epidemiological evaluation of the spreading of certain pathogens. In order to determine the physical status of genomes, several strategies are currently available. Chromosome polymorphisms can be visualised by pulsed field gel electrophoresis, whereas more subtle variation can be demonstrated by the detection of restriction fragment length polymorphisms, particularly in combination with Southern hybridisation (9, 20). Recently, the polymerase chain reaction (PCR) has been used for the determination of genetic differences among closely related microorganisms. Two basically different approaches have been presented, based

on the type of primer used during genotyping. Short oligonucleotide primers give rise to random amplification of polymorphic DNA at low stringency annealing conditions (RAPD analysis, references 2 and 24). Also, the application of repeat motif primers aiming at DNA domains which seem to vary among species in particular (interrepeat PCR) has been described (16). In this communication the genetic analysis of *G. duodenalis*, an intestinal protozoan parasite, by interrepeat PCR and RAPD analysis is described. Both isolates and cloned lines are included in the comparison.

MATERIALS AND METHODS

Origin and characterisation of *G. duodenalis* isolates: For the present study, most of the *G. duodenalis* isolates were collected from human subjects in Dutch hospitals (codes AMC and Nij). Some *G. duodenalis* infections had been contracted during visits to foreign countries, some in the Netherlands. Clinical data available are quite limited (6). All infections caused by the Nij-1 to -5 isolates were most probably contracted in the Netherlands. Ny-1, Nij-2 and Nij-3 caused symptomatic infections, the others caused a symptomatic infection. AMC strain 1-3 originated in Africa and only for strain AMC-1 a symptomatic infection has been documented. AMC-4 was isolated from a symptomatic patient in the Netherlands. AMC-6 (asymptomatic) and AMC-9 (symptomatic) were isolated in Nicaragua and India, respectively. The Australian isolate BAC1 (from the cat), established as an axenic culture from trophozoites, was kindly donated by B.P. Meloni (12). The Portland-I strain (13) was used as a reference isolate in some experiments.

Cloning of *G. duodenalis*: Cloning of parasites was performed as described before (3). In short, single microdrops of a diluted cell suspension (approx. 6-8 trophozoites per μl) were placed on coverslips and examined by microscopy. When only one organism was observed in the microdrop, the entire coverslip was aseptically immersed in a 4 ml cloning vial containing prewarmed medium. After 4-10 days, upon observation of dividing trophozoites, larger volume cultures were inoculated. Clones could either be cryopreserved (11) or processed for DNA isolation.

DNA isolation: DNA was isolated from parasites according to standard procedures (6). In short, parasites were suspended in 50 mM Tris.HCl pH 8.0, 0.5% SDS, 200 μg/ml proteinase K and incubated at 37°C for 18 h. Subsequently, phenol- and chloroform-isoamyl alcohol extractions were performed. After ethanol precipitation the nucleic acid pellet was dried and resuspended in distilled water. After an additional RNase A treatment, a second round of phenol extraction and ethanol precipitation the pellet was dissolved in 200 μl of 10 mM Tris.HCl, pH 8.0, 1 mM EDTA. Storage was at -20°C.

Oligonucleotides: The three interrepeat PCR primers used in this study were synthesised on an Applied Biosystems DNA synthesizer, model 387A. The sequences of primers 799, 855 and 944 were $(GTGTGGG)_3$, $(TAGGG)_5$ and $(GGA)_7$, respectively. Primers 799 and 944 have been described before as primers 9 and 4 (21). For RAPD

analysis primer 1 (5'CGGCGGGGCG3') was used. Primers were used directly, without prior purification by high performance liquid chromatography (HPLC).

Interrepeat PCR analysis: PCR reaction mixes (100 μl) contained 10 mM Tris.HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100 and 0.2 mM of all four deoxyribonucleotide triphosphates. Per reaction 0.5 units of Taq DNA polymerase (Sphaero Q, Leiden, The Netherlands) were included. The amount of template DNA used was approximately 100 ng per reaction and 50 pmol of a single repeat consensus primer (either 799, 855 or 944) was included in the amplification assay. Amplification was performed by cycling 40 times between denaturation, annealing and enzymatic chain extension temperatures in a Biomed thermocycler (model 60). Denaturation took place by incubation at 94°C for 1 min, annealing occurred at 52°C for 2 min, whereas enzymatic synthesis of DNA was performed at 74°C for 3 min. As controls, vials containing all ingredients either without template DNA or without primer were subjected to the same amplification procedures. Results were validated only in case the controls contained no amplified DNA. Amplified DNA was analyzed by agarose gel electrophoresis and ethidium bromide staining. Gels (15x20 cm) were run at 100 mA for 3 h and buffered at pH 7.8 in the presence of 40 mM Tris.acetate, 2 mM EDTA.

Amplification of random DNA segments: Analysis was performed essentially as described before (reference 25 and above). Reactions were carried out in 25 μl volumes using 25 ng of genomic DNA and 0.5 units Taq DNA polymerase. After 7 min of denaturation (92°C), 45 cycles consisting of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C each followed. Amplified DNA was analyzed as described above.

RESULTS

Interrepeat PCR of G. duodenalis isolates: In previous studies we employed PCR primers derived from eukaryotic telomeric and simple sequence motifs in order to generate species-specific DNA fingerprints for several lower eukaryote species (14, 21, 23). In all PCR-mediated assays, reproducibility is of major concern. All results were obtained by performing experiments under a very strict laboratory regime. DNA samples are purified, stored and aliquoted in a standardised manner. Thermocyclers were analyzed to exclude the possibility of positional amplification variability. Oligonucleotide primer batches were prepared in large stocks in order to warrant constant quality. Furthermore, it appeared that PCR fingerprinting of *G. duodenalis* DNA was relatively insensitive to variations in DNA template concentration. In the range between 100 ng to 0.5 ng DNA per reaction no major changes in the DNA fingerprints were detectable. Amplification was less efficient at the lower template concentrations, however. Also, purification of the crude primer batches by high performance liquid chromatography did not result in major changes in the fingerprinting patterns (results not shown).

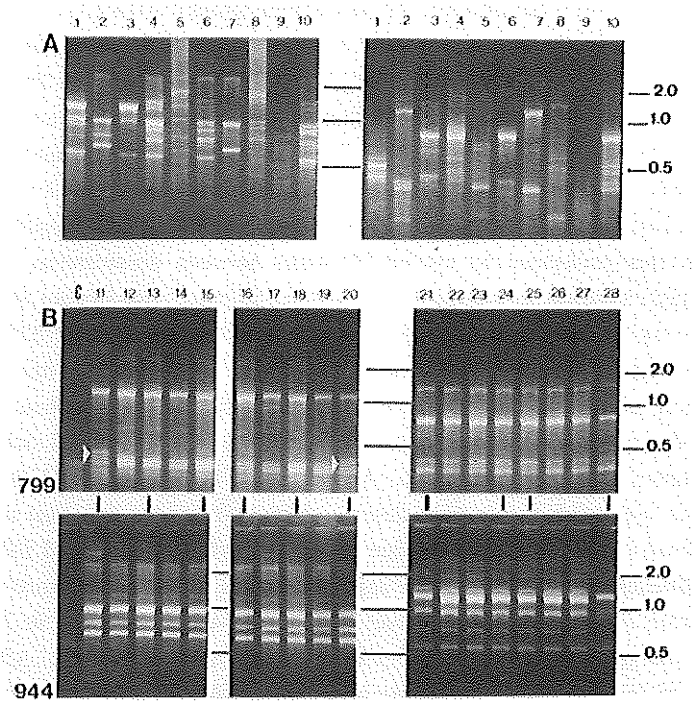


Figure 1 PCR fingerprinting of *G. duodenalis* isolates and clones using repeat consensus primers $(GGA)_7$ and (GTGTGGG)3 (primers 944 and 799). **(A).** DNA from 10 different *G. duodenalis* isolates has been amplified by interrepeat PCR. The panel on the left shows the results obtained with primer 944, on the right the results obtained by combining the same DNA samples with primer 799 are displayed. Numbering from 1 to 10 encodes isolates BAC-1, Nij-3, Nij-2, Nij-1, AMC-9, AMC-6, AMC-4, AMC-3, AMC-2 and AMC-1, respectively. The lack of amplified DNA in lane 9 (panel 799) may be due to insufficient amounts of template DNA, but it may be possible that DNA from this strain does not react with this primer: amplimers were obtained upon with primer 855 (Figure 2A, lane 5). **(B).** DNA from 2 different sets of *G. duodenalis* clones has been amplified by interrepeat PCR. The upper row (3 panels) gives an overview of the results obtained with primer 799, whereas in the lower row the results obtained with primer 944 are displayed. Numbers 11-18 show amplified DNA from a collection of clones originating from isolate Nij-5 (Nij5-M and Nij5-M1), lanes 21-27 display the amplicons deriving from clones originating from isolate Nij2 (Nij2-M). Lanes 11-20 contain material from Nij5-10, Nij5-9, Nij5-8, Nij5-7, Nij5-6, Nij5-4, Nij5-3, Nij5-1, Nij5-M1 and Nij5-M, respectively. From 21-28 clones Nij2-9, Nij2-8, Nij2-7, Nij2-6, Nij2-3, Nij2-2, Nij2-1 and Nij2-M have been analysed. In the two lefthand pictures in the upper row, two arrowheads indicate the additional DNA fragment synthesised with the Nij5-10 and Nij5-M DNA as template. The lane C displays the negative control: no template DNA was added. On the right markers are indicated in kb.

PCR fingerprinting of *G. duodenalis* isolates and clones thereof was performed using the three repeat-motif oligonucleotides 799, 944 and 855 and arbitrary primer 1. The results obtained with primers 799 and 944 are combined in Figure 1. It is obvious that, contrary to that found for *Naegleria fowleri* isolates (21), the banding patterns generated for the 10 *G. duodenalis* isolates studied differ considerably (Figure 1A). On the basis of the 20 fingerprints shown, all 10 isolates can be discriminated, although it has to be mentioned that the fingerprints for the AMC-2 isolate (Figure 1A, lanes 9) apparently are of a low quality. Interestingly, the banding patterns obtained using primer 944 for isolates BAC1 and Nij-2 (Figure 1A, lanes 1 and 3) and for isolates Nij-1 and AMC-6 (Figure 1A, lanes 4 and 6) are virtually identical. The use of primer 799 instead, allows clear discrimination of these two couples, although the patterns obtained for Nij-1 and AMC-6 are still quite similar. Furthermore, both primers are only able to detect small genetic differences between isolates Nij-3 and AMC-4 (Figure 1A, lanes 2 and 7). It appears that the discriminatory power of primer 799 exceeds that of primer 944. Using 799 none of the *G. duodenalis* DNA preparations renders a fingerprint which is identical to that of one of the others. Finally, it is noteworthy that several of the DNA samples generate DNA fragments of identical size upon amplification directed by either primer. For instance in case of 799, in several instances a prominent 0.9 kb fragment is generated (see lanes 3, 4, 6, and 10), whereas primer 944 gives rise to the synthesis of a DNA fragment just over 0.5 kb in length (lanes 1, 3, 4, 6 and 10). On the basis of this bandsharing a certain degree of relatedness among the isolates could be substantiated. The overlap is in agreement with grouping based on previous RAPD analyses (see below and reference 6). Based on the previous observations isolates 3, 4, 6 and 10 could form a cluster of isolates which are more closely related to each other than to one of the other isolates included in this study. This type of comparison could lead to the establishment or confirmation of phylogenetic relationships and can be very useful in epidemiological studies on protozoan parasites in general (see Discussion).

Interrepeat PCR of G. duodenalis clones: When clones, deriving from single *G. duodenalis* isolates, are analyzed in a similar way, another picture appears. In Figure 1B it can be seen that *G. duodenalis* clones show (near) identical PCR fingerprints and in this respect resemble *N. fowleri* isolates. Clones deriving from the Nij5 isolate (Figure 1B, lanes 11 to 20) cannot be distinguished using primer 944. Also, the clones generated from the Nij-2 isolate are identical, with the exception of parental isolate Nij-2M and clone Nij 2-6 (lane 28 and 24). These latter clones show minor DNA fragment length variation in the region just below 1.0 kb. Amplification of DNA from all clones and using primer 799 confirms the results as described above. It should be mentioned that the pattern in lane 28 (Figure 1, primer 799 and 944) should be identical to that in lane 3 (Figure 1A, primer 799 and 944, respectively) since DNA from the same isolate was used as template. There is slight variation in band intensity, the relevance of which is unclear. This may be due to small variations in DNA template concentrations or differences in the length of the

cultivation period. However, clone Nij5-10 and the parental isolate Nij5-M (lanes 11 and 20) stand apart from the other clones. In the 5-10 clone and the original isolate 5-M an additional DNA fragment appears, which has an approximate length of 0.4 Kb (see small arrowheads in Figure 1B). The described differences between isolates Nij-5M (lane 20) and Nij-5MI (lane 19) may be due to the fact that the latter isolate has been in culture for a somewhat extended period of time. In conclusion, it can be stated that primers 944 and 799 allow the straightforward differentiation of *G. duodenalis* isolates. Apparently, the clones are genetically more homogeneous and cannot be distinguished easily using this set of primers.

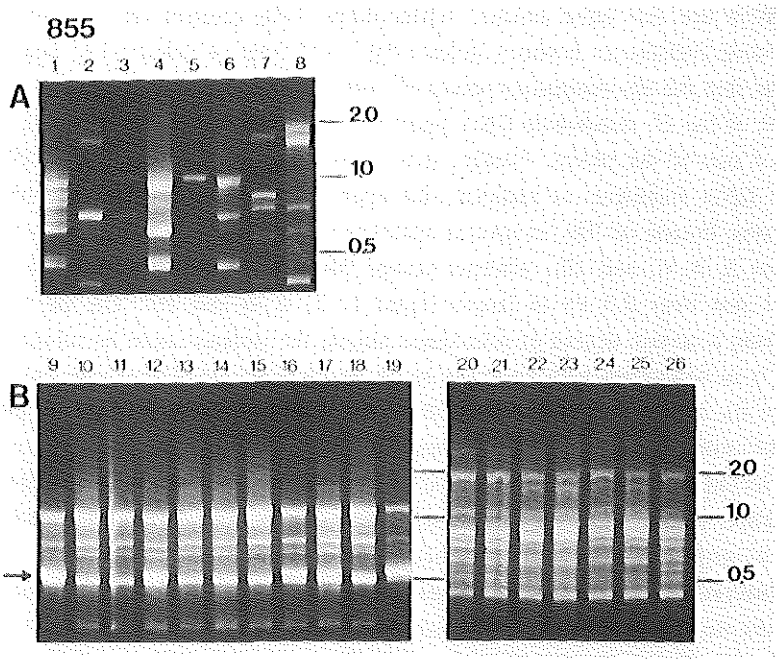


Figure 2 PCR fingerprinting of *G. duodenalis* isolates and clones using repeat consensus primer $(TAGGG)_5$ (primer 855). **(A).** Lanes 1-8 contain amplified DNA deriving from the *G. duodenalis* isolates AMC-9, AMC-6, AMC-4, AMC-3, AMC-2, Nij-3, Nij-2, and Nij-1 respectively. On the right molecular length markers are indicated in kb. **(B).** DNA from two different sets of *G. duodenalis* clones has been amplified using primer 855. From number 9-26 amplicons deriving from clones Nij5-10, Nij5-9, Nij5-8, Nij5-7, Nij5-6, Nij5-5, Nij5-4, Nij5-3, Nij5-1, Nij-5M1, Nij5-M, Nij2-9, Nij2-8, Nij2-7, Nij2-6, Nij2-2, Nij2-1 and Nij-2M are shown. The arrow on the left indicates the amplicon length polymorphism among the Nij-5 clones (lanes 9-19). On the right molecular length markers are indicated in kb.

Interrepeat PCR with the telomeric consensus sequence primer: Application of primer 855 in the PCR mediated genotyping of *G. duodenalis* isolates renders results comparable to those gathered with primers 799 and 944. In Figure 2A an example of the variation that can be detected among isolates is presented. Including primer 855 in the comparative analysis of *G. duodenalis* isolates further increases the discriminatory power of PCR fingerprinting by repeat motif primers. Even isolates AMC-6, Nij-2 and Nij-1 (lanes 2, 7 and 8, respectively, in Figure 3), producing comparable patterns with primers

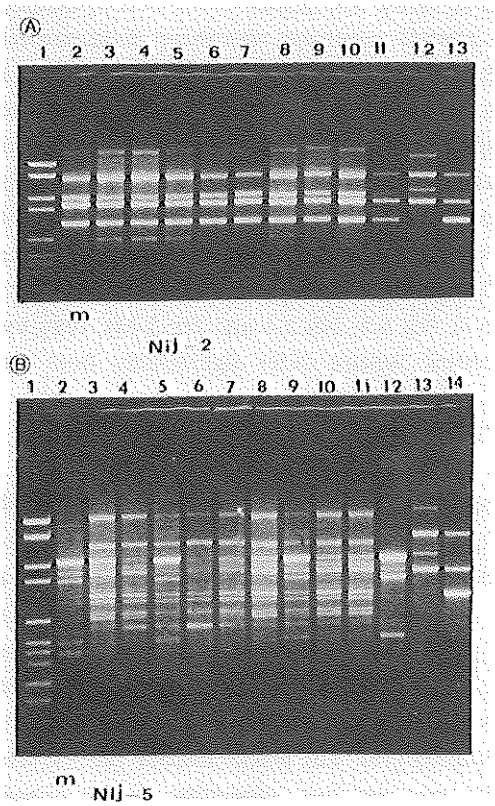


Figure 3 RAPD analysis of *G. duodenalis* clones using primer 1. **(A).** Lanes 2-13 contain amplified DNA deriving from clones Nij-2M, Nij2-1 to Nij2-10, BAC-1 and the Portland-I isolate, respectively. Lane 1 displays the pBr322 Pst digest, which serves as length marker. **(B).** Lanes 2-14 contain amplified DNA deriving from clones Nij-5M, Nij5-1 to Nij5-10, BAC-1 and the Portland-I isolate, respectively. Lane 1 displays the pBr322 Pst digest, which serves as length marker.

799 and 944, can easily be distinguished by primer 855. The analysis of amplified DNA deriving from the Nij-2 clones gives a complex picture. A large number of DNA fragments is generated (Figure 2B) and the resolution of the agarose gels used is not sufficient to allow a detailed interpretation of the picture. However, no major differences were apparent between the clones, except for an additional band for isolate Nij-2M (lane

26, 0.65 kb region). Amplification using primer 855 of DNA from the Nij-5 clones give a somewhat different result. In this case, two clearly different genotypes are encountered. Clones Nij5-10, Nij5-7, Nij5-3, Nij5-1 and Nij-5M give rise to a 0.5 kb DNA fragment which is not found in the six other clones (see arrow in Figure 2B). Note that Nij5-10 and Nij-5M, which gave a different DNA banding pattern when primer 799 was used, are again in the same cluster. Overall, primer 855 again allows discrimination between isolates of *G. duodenalis*. However, in contrast to PCR primers 799 and 944, primer 855 also allows the differentiation of one of two groups of clones into two genotypically different groups.

Random Amplification of Polymorphic DNA: The results of the RAPD analysis of the clonal *G. duodenalis* isolates are in general agreement with those of interrepeat PCR. The Nij-2 clones are highly homogeneous (Figure 3A), also Nij-2M (which could be identified separately by IR PCR using primers 799 and 944) is identical to the offspring clones. The BAC1 isolate stands apart from all Nij-2 clones while Portland I is highly similar. RAPD analysis of the Nij-5 clones corroborates the finding of heterogeneity as established using primer 855. The clones can be divided in two major fingerprint groups. These groups contain clones Nij 5-M, Nij 5-3, Nij 5-7 and Nij 5-10 on the one hand and all the other clones on the other. The first group shows significant homology to that defined by primer 855 (Nij 5-10, Nij 5-7, Nij 5-3, Nij 5-1 and Nij 5-M), only the Nij 5-1 clone differs. The BAC1 and Portland isolates differ significantly from the individual clones.

DISCUSSION

PCR can be used successfully for genetic characterisation of microorganisms. PCR with short arbitrary primers, about 10 nucleotides in length, can lead to fast and reproducible genetic typing. Even strains of a single species can be identified on the basis of a PCR generated DNA fingerprint (2, 24). We demonstrate here the feasibility of this strategy also for the species *G. duodenalis*. Beside arbitrary primers, primers aiming at variable tandem-repeat like loci are applied. Since this approach has proven to be useful for several other eukaryotic microorganisms (14, 16, 21, 23), genetic variation in *G. duodenalis* was monitored in a comparable fashion (14). The results indicate that isolates of *G. duodenalis* can be distinguished with ease and that, depending on the primer chosen, genetic variation can even be detected among clones originating from a single isolate. The latter phenomenon was also observed upon karyotyping of *G. duodenalis* clones (8, 9). Using PCR with primers 1 or 855, a large percentage of offspring clones deviates from the parental strain (compare Nij-5 clones to Nij-5M) but they are identical to one other.

Telomere variation in *G. duodenalis* can be monitored by hybridisation analyses with ribosomal probes, since the ribosomal operons are present as tandem repeats within telomeric regions (10). Interestingly, the use of primer 855 allows the detection of

significant differences between related clones by PCR (Figure 2B). Since this primer has a base composition representing a multimeric form of the *G. duodenalis* telomeric consensus repeat motif (TAGGG) (10), this observation corroborates the occurrence of frequent genetic rearrangements in the telomeric regions (9). However, 60% variation among clones, as has been documented using rRNA gene hybridisation or pulsed field gel electrophoresis (8, 9, 10) is not fully confirmed by the PCR results. It should also be mentioned that it is by no means clear whether or not primer 855 really amplifies telomeric regions. This may even be unlikely, since telomeric repeats are usually in a head-to-tail orientation, whereas PCR requires an inverted orientation of the two primer binding sites. Using PCR, 60% of offspring clones do deviate from the parental strain (compare Nij-5 clones to Nij-5M) but they are all identical. Apparently, the PCR using single repetitive primers scans a relatively small portion of the parasite genome. This makes the observation that a high degree of variability among different isolates is found, a notable one.

When the results presented for *G. duodenalis* are compared to those recently published for another protozoan parasite, the amoeba *Naegleria fowleri* (21), some interesting differences are observed. When DNA from several species belonging to the genus *Naegleria* is amplified using the simple sequence and yeast telomere primers 799 and 944, respectively, species specific DNA fingerprints can be generated. DNA samples from a large number of isolates of a single *Naegleria* species, however, give rise to identical PCR fingerprints. This implies that, when the species *N. fowleri* and *G. duodenalis* are compared, the latter species seems to be hypervariable with respect to the DNA loci amplified by this set of PCR primers. In that respect, *G. duodenalis* also deviates from several fungi that were recently studied using a similar approach (results not shown). So, using this particular genotyping assay, *N. fowleri* behaves like a very stable, world-wide uniform protozoan clone, whereas *G. duodenalis* displays extensive genetic variation. Within the discussion on clonal reproduction of parasitic protozoa (17, 18), it appears that, as measured by interrepeat PCR and RAPD assays, these two parasitic protozoan species seem to differ highly with respect to their mode of clonal reproduction. This finding is corroborated by the fact that isozyme typing again reveals identity among *N. fowleri* isolates (5) and diversity among *Giardia* strains (6). The major question is why *Naegleria* does not display the degree of variation monitored for *Giardia duodenalis* by both interrepeat PCR and isozyme typing. This question also concerns definition of a species: is *Giardia* a complex of (closely) related species (like the *Naegleria* species) or is the species *G. duodenalis* just genetically more flexible? An argument in favour of the latter option is the marked difference in ecological behaviour of the two species. *Naegleria* thrives in a fairly uniform environmental niche, whereas *G. duodenalis* needs the ability to adapt to different hosts, displaying different immunological responses. This could also explain the antigenic surface variability in *G. duodenalis* (1). On the other hand, in *Naegleria lovaniensis*, which is closely related to *N. fowleri* and

inhabits a comparable environmental niche, a high number of distinct genotypes has been detected, leading to the assumption that sexual reproduction occurs in this species (15). However, based on the results presented in this paper no definite conclusions can be drawn.

In conclusion, a procedure enabling PCR-mediated *G. duodenalis* strain or clone identification is presented. Using interrepeat PCR or RAPD analysis, different levels of variation can be monitored, especially when different protozoan organisms are compared. Among isolates of a single species, different degrees of genetic polymorphism can be identified, depending on PCR primer choice. It remains to be seen whether these levels of diversity can be correlated to phenotypic characteristics of the parasites or the nature of the habitat the parasite occupies.

REFERENCES

1. **Aggarwal, A. and T.E. Nash** (1988) Antigenic variation in *Giardia lamblia* in vivo. Inf. Immun. **56**: 1420-1423.
2. **Akopyanz, N., N.O. Bukanov, T.U. Westblom, S. Kresovich and D.E. Berg** (1992) DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR based RAPD fingerprinting. Nucleic Acids Res. **20**: 5137-5142.
3. **Binz, N., R.C.A. Thompson, B.P. Meloni and A.J. Lymbery** (1991) A simple method for cloning *Giardia duodenalis* from cultures and fecal samples. J. Parasitol. **77**: 627-631.
4. **Carle, G.F., M. Frank and M.V. Obron** (1986) Electrophoretic separation of large DNA molecules by periodic inversion of the electric field. Science **232**: 65-68.
5. **De Jonckheere, J.F** (1987) Characterisation of *Naegleria* species by restriction endonuclease digestion of whole cell DNA. Mol. Biochem. Parasitol. **24**: 55-66.
6. **Homan, W.L., F. van Enckevort, L. Limper, G. van Eijs, G. Schoone, W. Kasperzak, A. Majewska and F. van Knapen** (1992) Comparison of *Giardia* isolates from different laboratories by isoenzyme analysis and recombinant DNA probes. Parasitol. Rev. **78**: 316-323.
7. **Kemp, D.J., J.K. Thompson, D. Walliker and L.M. Corcoran** (1987) Molecular karyotype of *Plasmodium falciparum*: conserved linkage groups and expendable histidine rich protein genes. Proc. Natl. Acad. Sci. USA **89**: 7672-7676.
8. **LeBlanq, S., S.H. Korman and L.H.T. van der Ploeg** (1992) Spontaneous chromosome rearrangements in the protozoan *Giardia lamblia*: estimation of mutation rate. Nucleic Acids Res. **20**: 4539-4545.
9. **LeBlanq, S., S.H. Korman and L.H.T. van der Ploeg** (1991) Frequent rearrangements of rRNA encoding chromosomes in *Giardia lamblia*. Nucleic Acids Res. **16**: 4405-4412.
10. **LeBlanq, S., R.S. Kase and L.H.T. van der Ploeg** (1991) Analysis of *Giardia lamblia* rRNA encoding telomere with $(TAGGG)_n$ as the telomere repeat. Nucleic Acids Res. **19**: 5790.
11. **Lyman, J.R. and G.L. Marchin** (1984) Cryopreservation of *Giardia lamblia* with diethyl sulfoxide using a Dewar flask. Cryobiology **21**: 170-176.

12. **Meloni, B.P., A.J. Limbery and R.C.A. Thompson** (1988) Isoenzyme electrophoresis of 30 isolates of *Giardia* from humans and felines. Am. J. Trop. Med. Hyg. **38**; 6573.
13. **Meyer, E.A** (1976) *Giardia lamblia*: isolation and axenic cultivation. Exp. Parasitol. **39**: 101-105.
14. **Niesters, H.G.M., W.H.F. Goessens,J.F.G.M. Meis and W.G.V. Quint** (1993) Rapid, Polymerase Chain Reaction based identification assays for *Candida* species. J. Clin. Microbiol. **31**: 904-910.
15. **Pernin, P., A. Ataya and M.L. Cariou** (1992) Genetic structure of natural populations of free-living amoeba, *Naegleria lovaniensis*: evidence for sexual reproduction. Heredity **68**: 173-181.
16. **Riley, D.E., M. Samadpour and J.N. Krieger** (1991) Detection of variable DNA repeats in diverse eukaryotic microorganisms by a single set of polymerase chain reaction primers. J. Clin. Microbiol. **29**: 2746-2751.
17. **Tibayrenc, M., F. Kjellberg and F.J. Ayala** (1990) A clonal theory of parasitic protozoa: the population structures of *Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas* and *Trypanosoma* and their medical and taxonomical consequences. Proc. Natl. Acad. Sci. USA **87**: 2414-2418.
18. **Tibayrenc, M., F. Kjellberg, J. Armond, B. Cury, S.F. Breniere, M.L. Dorde, and F.J. Ayala** (1991) Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. Proc. Natl. Acad. Sci. USA **88**: 5129-5133.
19. **Turner, M.J** (1989) *Trypanosome* variant surface glycoprotein. In: The Biology of Parasitism, Eds. P. Englund and S. Sher. Alan R. Liss, Inc., New York, pp. 349-370.
20. **Upcroft, P., R. Mitchell and P.L. Boreham** (1990) DNA fingerprinting of the intestinal parasite *Giardia dudodenalis* with the M13 phage genome. Int. J. Parsitol. **20**: 319-323.
21. **Van Belkum, A., J. de Jonckheere and W. Quint** (1992) Genotyping *Naegleria* spp. and *Naegleria fowleri* isolates by interrepeat polymerase chain reaction. J. Clin. Microbiol. **30**: 2595-2598.
22. **Van Belkum, A., J. Ramesar, G. Trommelen and A.G. Uitterlinden** (1992) Mini- and micro-satellites in the genome of rodent malaria parasites. Gene **118**: 81-86.
23. **Van Belkum, A., W. Melchers, W.G.V. Quint and J.F.G.M. Meis** (1993) typing of Aspergillus and Aspergillus fumigatus isolates by interrepeat polymerase chain reaction. J. Clin. Microbiol. **31**: 2502-2505.
24. **Welsh, J. and M. McClelland** (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18**: 7213-7218.
25. **Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey** (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic marker. Nucleic Acids Res. **18**: 6531-6535.

Adapted from: J. Clin. Microbiol. 1994;32:2528-2532

CHAPTER V

MONITORING SPREAD OF *MALASSEZIA* INFECTIONS IN A NEONATAL INTENSIVE CARE UNIT BY PCR-MEDIATED GENETIC TYPING

Alex van Belkum, Teun Boekhout[1] and Ron Bosboom[2]

[1]Centraalbureau voor Schimmelcultures, Yeast Division, Julianalaan 67, 2628 BC Delft, the Netherlands. [2]Academic Hospital Leiden, Dept. Medical Microbiology, P.O. Box 9600, 2300 RC Leiden, the Netherlands.

SUMMARY

***Malassezia furfur* and *Malassezia pachydermatis* were isolated from newborn children and incubators in a neonatal intensive care unit. To assess whether persistence or frequent import of the organisms was the cause of the elevated incidence, genetic typing of the strains was performed by PCR-mediated DNA fingerprinting. By using PCR primers aimed at repeat consensus motifs, six different genotypes could be detected in a collection of six *M. furfur* reference strains. In the case of ten *M. pachydermatis* reference strains nine different genotypes were detected by three different PCR assays. None of these assays could document genetic differences among the clinical isolates of either *M. furfur* or *M. pachydermatis*. On the basis of these results it is concluded that within the neonatal intensive care unit longitudinal persistence of both an *M. furfur* and an *M. pachydermatis* strain has occurred and that *Malassezia* species can persist on incubator surfaces for prolonged periods of time. It can be concluded that PCR fingerprinting is a *Malassezia* typing procedure that is to be preferred over the analysis of chromosomal polymorphisms by pulsed-field gel electrophoresis in this genus.**

INTRODUCTION

The basidiomycetous yeast genus *Malassezia* presently consists of three species (27). *Malassezia furfur* and *Malassezia sympodialis* are obligatory lipophylic skin flora yeasts of humans, whereas *Malassezia pachydermatis* is a non-obligatory skin flora yeast also encountered in several other mammals (18). *M. furfur* can cause a relatively broad spectrum of clinical phenomena, varying from pityriasis versicolor in adults (1) to life threatening invasive disease in neonates (9, 11, 14, 19, 20, 24, 28, 29). There is also an increasing number of reports discussing the relevance and potential clinical problems of *Malassezia* infections in cancer (10, 26) or AIDS patients (27). Since *Malassezia* species are encountered in clinical diagnostics more frequently, the need for identification and typing assays will increase in the coming years.

For *M. furfur*, an immunological assay discriminating three serovars has been described (8). It is clear that this degree of resolution is insufficient for detailed epidemiological comparisons among clinical and environmental isolates. For epidemiological ty-

Table 1. Overview of *Malassezia* strains

M. furfur		
1. CBS 1878	R.W. Benham, type of *Pityrosporum ovale*	
2. CBS 7019	V.K. Hopsu neotype, pityriasis versicolor, Finland	
3. CBS 6094	F. Keddie, normal skin, USA	
4. CBS 4172	B.A. Gustafson, skin of eland	
5. CBS 5333	F. Blank, lesion of skin, Canada	
6. CBS 6000	I.G. Murray, ex dandruff, India	
7. AZL 113598	R. Bosboom, urine of neonate B,	11-05-90
8. AZL 112926	R. Bosboom, urine of neonate A,	06-05-90
9. AZL 115292	R. Bosboom, urine of neonate B,	28-05-90
10. AZL 132977	R. Bosboom, urine of neonate D,	26-11-90
11. AZL 133102	R. Bosboom, urine of neonate E,	27-11-90
12. AZL 112426	R. Bosboom, skin of neonate A,	30-04-90
13. AZL 113850	R. Bosboom, urine of neonate B,	13-06-90
M. sympodialis		
14. CBS 7222	R.B. Simons, type auditory tract, USA	
15. CBS 7709	E. Guley, skin with seborrheic excema, the Netherlands	
16. CBS 7705	E. Guley, pityriasis versicolor, the Netherlands	
17. CBS 7707	E. Guley, pityriasis versicalor, the Netherlands	
M. pachydermatis		
18. CBS 7044	R. van Breuseghem	
19. CBS 4165		
20. CBS 1891	J. Lodder, ear of dog, the Netherlands	
21. CBS 1884	B.A. Gustafon, ear of dog, Sweden	
22. CBS 6534	H.E. Rhoades, ear of dog, USA	
23. CBS 1919	P.W.C. Austwick, ulcerated ear of dog, UK	
24. CBS 6541	H.E. Rhoades, ear of dog	
25. CBS 6537	H.E. Rhoades, ear of dog, USA	
26. CBS 1885	J. Lodder, ear of dog, the Netherlands	
27. CBS 6542	H.E. Rhoades, ear of dog	
28. CBS 6535	H.E. Rhoades, ear of dog, USA	

29. AZL Jonge		19-03-91	30. AZL A4	incubator	22-01-91
31. AZL Wong		15-01-91	32. AZL D5	incubator	27-03-91
33. AZL KV		17-04-91	34. AZL 107498	NK	NK
35. AZL RV		26-02-91	36. AZL SH		19-03-91
37. AZL 101992	NK	NK	38. AZL ST		22-01-94
39. AZL D3	incubator	06-03-91	40. AZL CM		17-04-91
41. AZL WS		05-02-91	42. AZL JV		17-01-91

CBS: Centraalbureau voor Schimmelcultures, Yeast Division, Delft, the Netherlands; AZL: Academic Hospital Leiden, the Netherlands. The numbers 1 through 42 correspond to the numbering used in Figures 1 and 2. Isolation dates are given in day-month-year. (NK: date of isolation not known).

ping of yeasts in general various molecular procedures have been developed (23), but with respect to *Malassezia* typing only a small number of reports have appeared in recent literature. These mainly elaborate on karyotyping by pulsed field gel electrophoresis (4,13), which again does not allow for highly efficient discrimination of *Malassezia* strains. In order to discriminate the persistence of *Malassezia* strains from increased importation, novel procedures must be implemented. In this respect the PCR may prove to be valuable (22). PCR can be used for the detection of subtle DNA polymorphisms by application of primers that aim at hypervariable DNA loci (32, 33). This laboratory technique has already been used frequently in order to determine inter-isolate relationships among fungal and protozoal species (3, 7, 12, 15, 16, 21, 30, 31, 34). The present report describes the application of PCR fingerprinting for the resolution of *Malassezia* epidemics in a neonatal intensive care unit. Recently, within a neonatal intensive care unit at the Academic Hospital Leiden (the Netherlands), an increase in the number of *M. furfur* and *M. pachydermatis* infections was encountered (6). Several of the environmental and patient isolates were typed by PCR and epidemiological implications will be discussed. Since some of the strains have also been karyotyped by pulsed-field electrophoretic separation of chromosomes (4), a comparison between karyotyping and PCR genotyping can be performed.

MATERIALS AND METHODS

Yeast strains and paediatric patients: In the present study both *M. furfur, M. pachydermatis* and *M. sympodialis* strains were analyzed. *M. furfur* and *M. pachydermatis* were grown on Leeming and Notman agar (1% peptone, 0.5% glucose, 0.1% yeast extract, 0.4% desiccated ox bile, 0.1% glycerol, 0.05% glycerol monostearate, 0.05% Tween-60, 1% whole-fat cow's milk, 1.5% agar). In case of *M. furfur* cultivation, this medium was supplemented with a small amount of olive oil. *M. sympodialis* was grown on 1% yeast extract, 0.5% peptone, 4% glucose agar (YPGA medium). All strains studied were cultivated at 35°C (Table 1). Table 1 lists the reference isolates from the Centraalbureau voor Schimmelcultures CBS (Yeast Division, Delft, the Netherlands) and the clinical isolates collected in the departments of Neonatology or Dermatology at the Academic Hospital Leiden. Relevant patient data are included.

DNA isolation and PCR: To prevent contamination in the PCR assays, DNA isolation and amplification were performed in separate laboratory rooms. Transport of laboratory equipment between these rooms was strictly forbidden. Also, the PCR ingredients were stored in a separate room. In order to isolate DNA, cells were scraped from the culture media and collected in an appropriate volume of phosphate buffered saline pH 7.0 (PBS). Cells were pelleted by centrifugation, washed with another volume of PBS and centrifuged again. For the *M. pachydermatis* and *M. sympodialis* strains, effective lysis could be achieved by treating cells with Novozym (Novo Industries, A/S), subsequent lysis of spheroplasts and DNA purification by a fast method with guanidinium

Table 2 Survey of primers used for PCR fingerprinting of *Malassezia* strains

primer code	nucleotide sequence
ERIC IR	5'-ATGTAAGCTCCTGGGGATTCAC-3'
ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'
REP1R-I	5'-IIIICGICGICATCTGGG-3'
REP2-I	5'-ICGICTTATCIGGCCTAC-3'
BG-2	5'-TACATTCGAGGACCCCTAAGTG-3'

Note: All primers have been described previously (31, 32); I stands for the base derivative inosine.

isothiocyanate (5). Since the clinical *M. furfur* isolates seemed to resist this procedure, an alternative and more generally applicable procedure for isolation of DNA from *Malassezia* species was developed. Cell pellets were washed once more in distilled water and lyophilised for at least 60 h. The resulting powder was resuspended in a buffer containing 100 mM Tris.HCl pH 6.4, 4 M guanidinium isothiocyanate, 10 mM EDTA, 2% (v/v) Triton X-100 and the mixture was incubated at 37°C for at least two h. Thereafter, DNA was purified by affinity chromatography (5). The amounts of isolated DNA were estimated by electrophoresis in 1% agarose gels in 40 mM Tris.Borate pH 7, 1 mM EDTA (0.5xTBE) and comparison with ethidium bromide stained amounts of bacteriophage lambda DNA (Promega) in a parallel run.

For PCR, approximately 50-100 ng of *Malassezia* DNA was dissolved in a 100 µl volume containing 10 mM Tris.HCl pH 9, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM deoxyribonucleotide triphosphates and 0.5U Taq DNA polymerase (Sphaero Q, Leiden, the Netherlands). After addition of 50 pmoles of one or more of the PCR primers surveyed in Table 2, the aqueous phase was overlaid with mineral oil and thermocycling was performed in a Biomed type 60 PCR machine. The PCR programme consisted of a 4 min predenaturation step at 94°C, 40 cycles of 1 min 94°C, 1 min 25°C, 2 min 74°C and a final extension step of 4 min 74°C. Amplimers were analyzed by electrophoresis through 1-3% agarose gels in 0.5xTBE. Ethidium bromide stained gels were photographed by using Polaroid Polapan T52 pictures. PCR results were validated only when the negative control samples (in which no extraneous DNA was included) were demonstrated not to contain DNA after PCR.

RESULTS

All strains listed in Table 1 were subjected to various PCR assays. Initial experiments with the arbitrary primer BG2 (31) appeared to be unsuccessful, although this primer has been very useful in elucidating interrelationships among isolates of other fun-

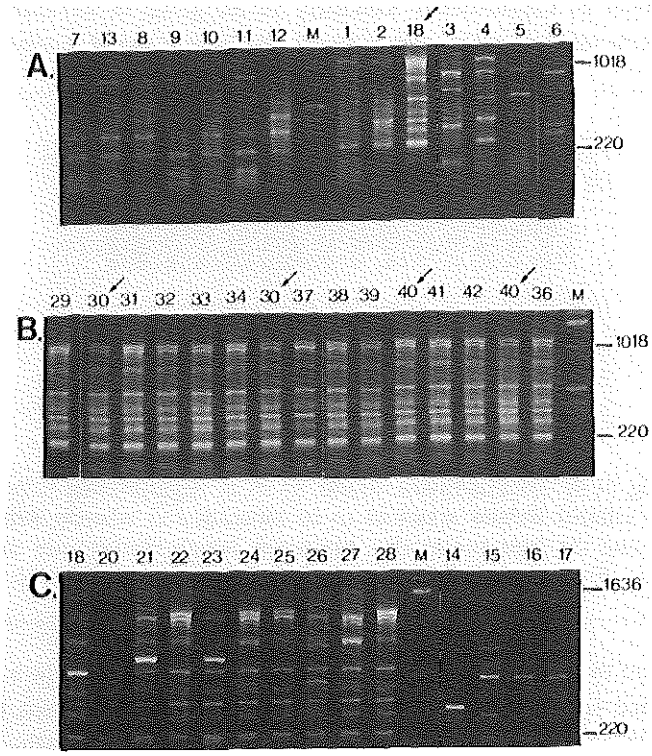


Figure 1 Typing of *Malassezia* strains by PCR fingerprinting with primers ERIC1 and ERIC2. **(A).** Comparison of clinical isolates (strains 7-13) and reference strains (strains 1-6) of *M. furfur*. The banding patterns indicate homogeneity among the clinical isolates, versus genetic heterogeneity among the reference strains. Note that clinical isolates 9 and 11 display minor differences when they were compared with the other patient strains (see also Results). The isolate indicated mp (arrow) represents *M. pachydermatis*. **(B).** PCR analysis of clinical *M. pachydermatis* strains. Strains 30 and 40 were run in duplicate (arrows). All isolates display identical DNA banding patterns. **(C).** PCR analysis of *M. pachydermatis* type strains (strain 18 and strains 20 to 28). DNA isolated from strain 20 appeared to be of poor quality. Strains 14 to 17 are *M. sympodialis*; strain 14 gave a clearly abberant DNA banding pattern. Molecular length markers (M) are indicated on the right in basepairs. Numbering of strains is as in Table 1.

gal species. The gels showed monomorphic DNA banding patterns of low complexity because only a few DNA fragments were synthesised (data not shown). Pilot experiments involving a small number of *Malassezia* strains revealed that the application of single REP or ERIC primers (32) was not highly discriminatory. However, the combination of primers ERIC IR and ERIC2 in a single reaction revealed a sufficiently high degree of DNA diversity among the various strains. As can be seen in Figure 1, all six CBS strains of *M. furfur* could be unequivocally identified. In the group of ten CBS strains of *M. pachydermatis* nine different types were detected. In Table 3, the *M. pachydermatis* geno-

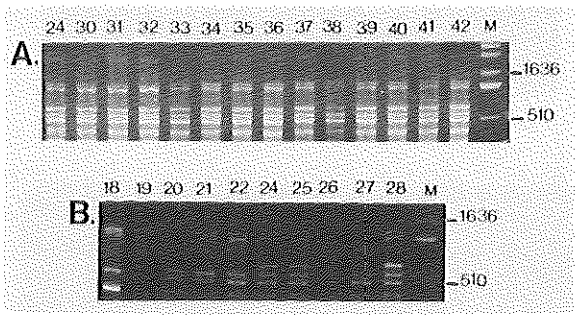


Figure 2 Typing of *M. pachydermatis* strains by PCR fingerprinting with primers REP1 and REP2. All clinical isolates appeared to be identical (A) whereas heterogeneity was encountered among the type strains (B). Molecular length markers are indicated on the right in basepairs. For indexing of the banding patterns for the type strains, Table 3.

codes constructed by identifying given DNA fingerprints with a letter or a number, show that the combined results of two PCR assays allowed nine of ten strains to be discriminated. Only CBS 6541 and CBS 6535, both American isolates originating from the dog auditory tract, could not be discriminated. In order to gain insight in the genetic diversity that can be highlighted by PCR fingerprinting for this *Malassezia* species, more isolates, preferably from multiple geographic origins, must be analyzed.

All *M. sympodialis* reference strains could be distinguished on the basis of their DNA fingerprints. It was shown that the banding pattern of CBS7222, the type strain of *M. sympodialis* was grossly different from those of the other strains. All clinical *M. pachydermatis* strains appeared to be identical (Figure 1). Interestingly, all incubator isolates (AZL-A4, AZL-D5 and AZL-D3) were genetically comparable. This implies that despite regular cleaning of the incubators, *M. pachydermatis* persists on the glass surfaces. Since the isolation dates were over two months apart, this persistence may be clinically relevant. Apparently, the clinical *M. furfur* strains are clonally related, be it that strains AZL 115292 and AZL 133102 displayed minor differences with regard to the

other hospital isolates when the combination of the ERIC primers was used. These slightly aberrant strains were derived from two different patients. The differences among the clinical isolates of *M. furfur* are much smaller than those among the CBS reference strains. The combination of REP-IR1 and REP2-I primers generated useful results, especially for the *M. pachydermatis* strains. However, PCR fingerprints for *M. furfur* and *M. sympodialis* were relatively vague, but the results described above were supported. PCR with REP1/REP2 did not discriminate among the clinical *M. furfur* strains. Figure 2 shows the results for *M. pachydermatis* type strains and clinical isolates.

Table 3 PCR fingerprinting genocodes for the CBS strains of *M. pachydermatis*

CBS number	number in table 1 and fig. 1,2	PCR assay ERIC1 ERIC2	PCR assay REP1 REP2	PCR assay OVERALL TYPE
CBS7044	18	A	A	I
CBS1891	20	B	B	II
CBS1884	21	C	C	III
CBS6534	22	D	D	IV
CBS1919	23	E	E	V
CBS6541	24	F	F	VI
CBS6537	25	D	F	VII
CBS1885	26	G	G	VIII
CBS6542	27	H	H	IX
CBS6535	28	F	F	VI
		8 types	8 types	9 types

Note: Both assays identify 8 types within the group of ten strains. Combination of the results only leaves strain 6541 and 6535 undiscriminated.

DISCUSSION

Whereas several genomic typing procedures for *Malassezia* yeasts appeared to be insufficiently discriminatory, PCR fingerprinting can be successfully used for adequate typing of *Malassezia* strains. Although the isolation of DNA from some strains may be troublesome and not all PCR primers generate sufficiently variable DNA banding patterns, several epidemiological useful assays can be developed by including multiple, prokaryotic repeat consensus primers like the ERIC or REP motifs. DNA can be isolated successfully by combining lyophilisation with the lytic activity of the chaotropic salt guanidinium isothiocyanate. Sufficiently discriminative assays can be designed.

As demonstrated in this report, on the basis of the homogeneity of the fingerprints of all clinical *Malassezia* isolates as opposed to the variation observed in groups of reference strains, it must be concluded that in the Neonatology ward of the Academic Hospital Leiden genuine nosocomial epidemics of both *M. furfur* and *M. pachydermatis* have occurred. This is corroborated by the fact that the *M. pachydermatis* isolates found to colonize the incubators were genetically indistinguishable over time. Also, *M. furfur* could be found on incubator surfaces. For this species it has been demonstrated that genotypically homologous strains could be isolated from the same surfaces three months after the original culture was obtained (data not shown). This phenomenon indicates that regular hygienic measures do not adequately remove or kill *Malassezia* yeasts.

Several of the strains described in this paper were karyotyped by pulsed-field gel electrophoresis (4). From the results of these studies it appears that the species *M. pachydermatis*, *M. furfur* and *M. sympodialis* could be identified on the basis of their characteristic karyotype, also showing that all species harbour a relatively small genome when compared with the genome sizes of other yeast species (35). Neither of the clinical *M. furfur* isolates could be discriminated on the basis of chromosomal length polymorphism, whereas the different CBS type strains of *M. furfur* could be divided in four distinguishable karyotypes. This type of DNA heterogeneity is in full agreement with the PCR results. The results obtained for *M. pachydermatis* seem to be contradictory. On the basis of length polymorphisms found in the smallest chromosomes, it has been suggested that the clinical isolates are diverse and that reinfection rather than an epidemic has taken place in the Neonatology ward (4). The PCR fingerprinting results, however, seem to indicate a genuine epidemic. In order to explain this contradiction, it could be assumed that the chromosomal length variation as observed in the clinical *M. pachydermatis* isolates is due to copy number variation in the repetitive ribosomal operons. This corroborates previously observed phenomena which reflect only a minor single-locus variability, which is generally considered a result of growth conditions. Amplification of ribosomal operons as a consequence of environmental factors has been demonstrated before (2, 17, 25) and cannot be used as a marker for overall genetic variability. The more dispersed type of multilocus variation detected by PCR may give a more global picture of genomic evolution. As a consequence the results of PCR fingerprinting of

Malassezia DNA may have more extensive epidemiological value than the detection of chromosomal length variation by pulsed-field gel electrophoresis.

REFERENCES

1. **Akpata, L.E., H.C. Gugnoin, R. Srivastava, and S.J. Utsalo** (1992) Dermatomycoses among industrial workers in Cross River State, Nigeria. Mycoses **35**: 371-374.
2. **Asakura, K., S. Iwaguchi, M. Homma, T. Sukal, K. Higashide, and K. Tanaka** (1991) Electrophoretic karyotypes of clinically isolated yeasts of *Candida albicans* and *C. glabrata*. J. Gen. Microbiol. **137**: 2531-2538.
3. **Aufauvre-Brown, A., J. Cohen, and D.W. Holden** (1992) Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. **30**: 2991-2993.
4. **Boekhout. T., and R.W. Bosboom** (1995) Karyotyping of *Malassezia* yeasts: taxonomic and epidemiological implications. Syst. Appl. Microbiol. **17**,146-153.
5. **Boom, R., C.J.A. Sol, M.M.M.Salimans, C.L. Jansen, P.M.E. Wertheim-van Dillen, and J. Van der Noordaa** (1990) Rapid and simple method for purificcation of nucleic acids. J. Clin. Microbiol. **28**: 495-503.
6. **Bosboom, R.W., G.W. Van der Kamp, and P.L.G. Petit** (1993) *Malassezia* spp. epidemic in a neonatal intensive care unit. Proc. Abstr. VI Eur. Congr. Clin. Microbiol. Infect. Dis. Sevilla: Abstr. 509.
7. **Cancilla, M.R., J.B. Powell, A.J. Hillier, and B.E. Davidson** (1992) Rapid genomic fingerprinting of *Kluyveromyces lactis* strains by arbitrarily primed polymerase chain reaction with ^{32}P and fluorescence labels. Appl. Environ. Microbiol. **58**: 1772-1775.
8. **Cunningham, A.C., E. Ingham, and G. Gowland** (1992) Humoral response to *Malassezia furfur* serovars A, B and C in normal individuals of various ages. Br. J. Dermatol. **127**: 476-81.
9. **Dankner, W.M., S.A. Spector, J. Furer, and C.E. Davis** (1987) *Malassezia* fungemia in neonates and adults: complication of hyperalimentation. Rev. Infect. Dis. **9**: 743-753.
10. **Francis, P., and T.J. Walsh** (1992) Approaches to management of fungal infections in cancer patients. Oncology **6**: 133-148.
11. **Gueho, E., R.B. Simmons, W.R. Pruitt, S.A. Meyer, and D.G. Ahearn** (1987) Association of *Malassezia pachydermatis* with systemic infection of humans. J. Clin. Microbiol. **25**: 1789-1790.
12. **Kersulyte, D., J.P. Woods, E.J. Keath, W.E. Goldman, and D.E. Berg** (1992) Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. J. Bacteriol. **174**: 7075-7079.
13. **Kiuchi, A., S. Takaraguchi, R. Hanizawa, M. Hara, T. Ikeda, and K. Tabuci** (1992) Chromosome sized DNA of *Malassezia pachydermatis* by pulsed field gel electrophoresis. J. Vet. Med. Sci. **54**: 1219-1220.
14. **Larocco, M., A. Dorenbaum, A. Robinson, and L.K. Pickering** (1988) Recovery of *Malassezia pachydermatis* from eight infants in a neonatal intensive care nursery: clinical and laboratory features. Pediatr. Infect. Dis. J. **7**: 398-401.

15. **Lehman, P.F., D. Lin, and B.A. Lasker** (1992) Genotypic identification and characterisation of species and strains within the genus *Candida* by using random amplified polymorphic DNA. J. Clin. Microbiol. **30**: 3249-3254.
16. **Loudon, K.W., J.P. Burny, A.P. Coke, and R.C. Matthews** (1993) Application of PCR to fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA. J. Clin. Microbiol. **31**: 117-21.
17. **Maleszka, R., and G.D. Clark-Walker** (1989) A petite strain of *Kluyveromyces lactis* has a 300 kb deletion in the rDNA cluster. Curr. Genet. **16**: 429-432.
18. **Marcon, M.J., and D.A. Powell** (1992) Human infections due to *Malassezia* spp. Clin. Microbiol. Rev. **5**: 101-119.
19. **Mickelsen, P.A., M.C. Viano-Paulson, D.A. Stevens, and P.S. Diaz** (1988) Clinical and microbiological features of infection with *Malassezia pachydermatis* in high risk infants. J. Infect. Dis. **157**: 1163-1168.
20. **Nichols, J.M., K.Y. Yuen, H. Saing** (1993) *Malassezia furfur* infection in a neonate. Br. J. Hosp. Med. **49**: 425-427.
21. **Niesters, H.G.M., W. Goessens, J.F.G.M. Meis, and W.G.V. Quint** (1993) Rapid PCR based identification assays for *Candida* species. J. Clin. microbiol. **31**: 903-908.
22. **Persing, P.H., and M.L. Landry** (1989) In vitro amplification techniques for the detection of nucleic acids: new tools for the diagnostic laboratory. Yale J. Biol. Med. **62**: 159-71.
23. **Pfaller, M.A** (1992) The use of molecular techniques for epidemiologic typing of *Candida* species. Curr. Topics Med. Mycol. **4**: 43-63.
24. **Richet, H.M., M.M. McNeil, M.C. Edwards, and W.R. Jarvis** (1989) Cluster of *Malassezia furfur* pulmonary infections in a neonatal intensive care unit. J. Clin. Microbiol. **27**: 1197-1200.
25. **Rustchenko-Bulgac, E.P** (1991) Variations of *Candida albicans* electrophoretic karyotypes. J. Bact. **173**: 6586-6596.
26. **Samonis, G., and D. Baljavoukas D** (1992) Fungal infections in cancer patients: an escalating problem. In Vivo **6**: 183-193.
27. **Simmons, R.B., and E. Gueho** (1990) A new species of *Malassezia*. Mycol. Res. **94**: 1146-1149.
28. **Stuart, S.M., and A.T. Lane** (1992) *Candida* and *Malassezia* as nursery pathogens. Semin. Dermatol. **11**: 19-23.
29. **Surmont, I., A. Gavilenes, J. Vandepitte, and E. Eggermont** (1989) *Malassezia furfur* fungaemia in infants receiving intravenous lipid emulsions. A rarity or just underestimated? Eur. J. Pediatr. **148**: 435-438.
30. **Van Belkum, A., J.F. De Jonckheere, and W.G.V. Quint** (1992) Genotyping of *Naegleria* spp. and *N. fowleri* isolates by interrepeat PCR. J. Clin. Microbiol. **30**: 2595-2598.
31. **Van Belkum, A., W.G.V. Quint, B. De Pauw, W. Melchers, and J.F.G.M. Meis** (1993) Typing of *Aspergillus* species and *Aspergillus fumigatus* isolates by interrepeat PCR. J. Clin. Microbiol. **31**: 765-769.
32. **Versalovic, J., T. Koeuth, and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19**: 6823-6831.

33. **Welsh, J., and M. McClelland** (1991) Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combination of primers. Nucleic Acids Res. **19**: 275-279.

34. **Woods, J.P., D. Kersulyte, W.E. Goldman, and D.E. Berg** (1993) Fast DNA iaolstion from *Histoplasma capsulatum*: methodology for arbitrary primed PCR-based epidemiological and clinical studies. J. Clin. Microbiol 31:463-464.

35. **Yoganathan, T., H. Lind, and G.A. Buck** (1989) An electrophoretic karyotype and assignment of ribosomal genes to resolved chromosomes of *Pneumocystis carinii*. Mol. Microbiol. **3**: 1473-1480.

Adapted from: J.Infect. Dis. 1994;169:1062-1070

CHAPTER VI

GENOTYPIC CHARACTERIZATION OF SEQUENTIAL *CANDIDA ALBICANS* ISOLATES FROM FLUCONAZOLE-TREATED NEUTROPENIC PATIENTS

Alex van Belkum, Willem Melchers[1], Ben E. de Pauw[2], Stewart Scherer[3], Wim G.V. Quint[4], and Jacques F. Meis[1]

[1]Department of Medical Microbiology and [2]Department of Hematology, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. [3]Lawrence Berkely Laboratory, Human Genome Center MS 74/157, 1 Cyclotron Road, Berkeley, Ca 94720, U.S.A. [4]Department of Molecular Biology, Diagnostic Center SSDZ, Reinier de Graaf Gasthuis,P.O. Box 5010, 2600 GA Delft, The Netherlands.

SUMMARY

A polymerase chain reaction (PCR)-mediated genotyping assay for *Candida albicans* has been developed. By amplification of genomic regions bordered by eukaryotic or prokaryotic repeat-like motifs, differences between individual *C. albicans* isolates can be determined. The resolution of this typing procedure is at least as good as that of other genotypic assays. To ascertain the epidemiologic and clinical usefulness of this PCR genotyping, a retrospective analysis of serial *C. albicans* isolates from neutropenic adults treated with fluconazole was done. By PCR genotyping, 40 strains were detected in 24 patients. Eighteen *C. albicans* strains were found on multiple samplings in individual patients. It appears that most patients remain colonised with a *C. albicans* strain of constant genotypic characteristics. However, exceptions were observed. In 7 (29%) of 24 patients, strains deviating from the most frequently encountered type could be identified. All but one strain remained susceptible to fluconazole in vitro after treatment in vivo. It was not possible to demonstrate a relation of genotypic variation and antifungal susceptibility changes.

INTRODUCTION

Fungal infections are a major threat for immunocompromised patients (17, 24). Despite a large array of preventive measures, patients cannot be protected completely from infection by pathogenic molds or yeasts. The most frequently encountered clinical problem is caused by *Candida albicans*. Many people are colonised with this yeast as a commensal organism. If persons become immunocompromised, their impaired immunological responses can enable the organism to establish deep-seated infections (8).

For this reason, immune-suppressed patients must be strictly monitored for the clinical presence of yeasts or molds. Appropriate surveillance can allow adequate diagnosis and early treatment of local or systemic infection. However, therapy is hampered by the limited number of well-evaluated fungostatic compounds or their associated nephrotoxicity. This implies that patients usually cannot be cleared of the fungal infection, only that problematic yeast growth can be suppressed. This poses the question whether chronic treatment with fungostatic agents imposes selection or mutagenic

generation of drug-resistant fungal strains. The latter would severely hamper the effectiveness of a compound in the eradication of new or recurrent infections. Genetic typing of fungal strains could demonstrate variability as a response to fungostatic treatment and as such could be useful for appropriate drug application.

Several DNA typing assays for pathogenic fungi have been described (3, 10, 13, 16). These vary from the direct detection of restriction fragment length polymorphisms (RFLP) to DNA probe-mediated analysis of polymorphic DNA. Recently, the polymerase chain reaction (PCR) has been adapted to detect interstrain variation in eukaryotic unicellular microorganisms (12, 15, 31). The methods are based on the random amplification of polymorphic DNA (RAPD analysis) by using short, arbitrarily chosen DNA primers. Otherwise, the procedures may be based on the detection of genomic regions known to be variable among lower eukaryotes, that is telomeric or repetitive DNA (interrepeat [IR] PCR). Both approaches have been applied for the genotypic characterization of a multitude of both bacterial and eukaryotic species and isolates (4, 30, 33).

The aim of the present study was to develop and use IR PCR for the genetic typing of *C. albicans*. A set of well-characterised *C. albicans* strains was subjected to a number of different IR PCR assays, the results of which were subsequently compared with those of other molecular typing techniques. Serial isolates of *C. albicans* were obtained from neutropenic patients, which were intermittently treated with fluconazole. The nature of the colonizing strain(s), the longitudinal analysis, and the effects of fluconazole on the *C. albicans* strains in certain patients will be discussed.

MATERIALS AND METHODS

***C. albicans* strains and patients:** *C. albicans* type strains (n=21) were obtained from the Lawrence Berkeley Laboratory (Berkeley, California, USA). These strains were previously studied by restriction fragment length polymorphism analysis, by Southern blotting with a probe specific for a *C. albicans* specific hypervariable DNA fragment and by pulsed field gel electrophoretic (PFGE) chromosome typing (13, 25). Clinical isolates of *C. albicans* were obtained from 24 neutropenic patients admitted to the University Hospital Nijmegen (the Netherlands). Patients were monitored for oral or fecal *C. albicans* carriage throughout their period of hospitalization. Incidental bloodstream or sputum isolates were included. In total, 117 yeast strains were collected (1-10 isolates per patient). Yeast isolates were identified as *C. albicans* on the basis of germ tube formation in human serum and by the production of chlamydospores on cornmeal agar. The strains and isolates were stored in glycerol-containing (50%) media at -80°C. Before genotyping, cells were grown for 48 h at 35°C on Sabouroud agar. During the collection period, most patients were treated for *C. albicans* infection (Table 1) with fluconazole (Roerig-Pfizer, Rotterdam (the Netherlands); 150 mg once daily for 2 weeks.

Susceptibility testing: MICs of fluconazole were determined by broth microdilution in 96-well microtiter trays as previously described (28). In brief, *C. albicans* isolates

were subcultured for 48 h at 35°C before use. Suspensions of 0.5 McFarland standard in 0.9% saline, spectrophotometrically adjusted to a turbidity that permits 75% transmission of light at 530 nm, were prepared. Before testing, the suspensions were diluted 1:100 in RPMI 1640 with L-glutamine in 0.165 M morpholinopropanesulfonic acid (MOPS; Sigma, Bornem, Belgium) (pH 7.0) providing working inocula of 10^4 to $5x10^4$ CFU/ml. Using a stock solution of 2000 μg/ml of fluconazole, 10-fold dilutions in duplicate (end concentration ranges from 0.05-100 μg/ml) were made with RPMI. Final yeast concentration was $5x10^3$-$5x10^4$ CFU/ml. A resistant strain (R 89-214; provided by M. Rinaldi, University of Texas Health Science Center, San Antonio) and a susceptible strain (AZN-1, University Hospital Nijmegen) of *C. albicans* were included. Inoculated trays were incubated at 35°C and read after 24h.

DNA isolation: *C. albicans* strains and isolates were grown on agar plates, and approximately 100 colonies were resuspended in 10 ml of phosphate buffered saline pH 6.8 (PBS). Of this suspension, 0.5 ml was centrifuged and the pellet was resuspended in 0.5 ml of 25 mM K_2HPO_4 pH 7.8, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 1 M sorbitol containing 0.1 mg/ml Zymolyase (Sigma, Bornem, Belgium). The resulting mixture was incubated at 37°C for 90 min and spheroplasts were lysed by the addition of 10 volumes of 50 mM Tris.HCl pH 6.4, 10 mM EDTA, 1% Triton X-100, 4M guanidinium isothiocyanate. After 10 min of incubation at room temperature, 100 μl of a Celite suspension (200 mg/ml) was added, and DNA isolation was done as described before (2). Finally, DNA was dissolved in 200 μl 10 mM Tris.HCl pH 8.0, 1 mM EDTA and stored at -20°C.

PCR genotyping: To establish the discriminatory power of PCR genotyping, eight different reactions were done on the DNA preparations from the reference strains. Essentially, two groups of four reactions, involving either eukaryotic or prokaryotic repeat primers, were carried out (Table 2 surveys the primer sequences). Reaction mixtures (100 μl) consisted of 10 mM Tris.HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM deoxyribonucleotide triphosphates, 50 pmol of primer(s), and 0.5 units of Taq DNA polymerase (Super Taq, Sphaero Q, Leiden, The Netherlands). Template DNA (50 ng) was added in a 5 μl volume. PCR was done in a thermocycler (Biomed model 60, Theres, Germany) using either of two programmes. The eukaryotic repeat primers were combined with a program of 40 cycles of 1 min at 94°C, 2 min at 52°C and 3 min at 74°C. The prokaryotic consensus motifs required a program consisting of 35 cycles of 1 min at 94°C, 1 min at 25°C, 2 min at 74°C, preceded by a 5 min incubation at 94°C and followed by a 10 min extension step at 74°C. Amplified DNA was separated by electrophoresis in agarose gels containing 1 μg/ml ethidium bromide. Banding patterns were counted and interpreted by visual inspection. To study the reproducibility of the assays, multiple colonies from each of several strains and isolates, were processed. Various cultures of the same strain or isolate were typed by PCR.

TABLE 1 SURVEY OF CLINICAL AND GENOTYPIC DATA OF *C. ALBICANS* STRAINS DERIVED FROM NEUTROPENIC PATIENTS BEFORE AND AFTER FLUCONAZOLE TREATMENT STUDIED BY PCR GENOTYPING

P	sample nr	material or treatment	sampling date	genotype I/II/III	MIC	P	sample nr	material or treatment	sampling date	genotype I/II/III	MIC
A	6541	ms/ts	07-16-92	A/A/A	0.2	N	4135	ms	07-15-90	C'/P/O	0.2
	6577	f	07-20-92	A/A/A	0.2			fluconazole	07-27-90		
	6687	ms/ts	08-13-92	A/A/A	0.2		4346	f	08-20-90	C'/P/O	0.2
	6688	f	13-13-92	A/A/A	0.2			fluconazole	08-08-90		
	6783	ms	08-29-92	A/A/A	0.2		4368	ms/ts	08-28-90	C'/P/O	0.2
	6784	f	08-29-92	A/A/A	0.2						
						O	6372	ms/ts	06-10-92	C/ND/A	0.2
B	4963	ms/ts	03-15-91	B/B/B	0.2		6373	f	06-10-92	C/ND/A	
	5242	ms/ts	06-24-91	B/B/B	0.4			fluconazole	06-10-92		
		fluconazole	07-02-91				6447	ms/ts	06-22-92	C/ND/A	0.2
		fluconazole	11-25-91				6455	f	06-22-92	C/ND/A	0.2
	6256	ms/ts	07-07-92	B/B/B	0.4		6537	ms/ts	07-16-92	C/ND/A	0.2
	6277	f	05-14-92	B/B/B	0.4			fluconazole	07-16-92		
	6278	ms/ts	05-14-92	B/B/B	0.2		6953	?	10-08-92	C/ND/A	0.2
	6461	f	06-29-92	B/B/B	0.4		6956	f	10-08-92	C/ND/A	0.2
	6462	ms/ts	06-29-92	B/B/B	0.4		7013	ms	10-26-92	C/ND/A	0.2
							7031	f	10-27-92	C/ND/A	0.4
C	4983	ms/ts	03-19-91	C/C/C	0.4			fluconazole	10-28-92		
	5031	f	04-03-91	C/C/C	0.4		7038	ms/ts	11-02-92	C/ND/A	0.2
		fluconazole	04-15-91								
	5085	f	04-29-91	C/C/C	0.4	P	4289	f	08-08-90	B'/C/A	0.2
							4290	ms/ts	08-08-90	B/H/O	0.2
D	5909	f	12-27-91	D/D/D	0.2		4433	f	09-24-90	B/H/O	<0.05
	5910	ms/ts	12-27-91	D'/E/E	0.2			fluconazole	10-04-90		
	6009	ms/ts	01-29-92	D/D/D	0.4		4583	ms/ts	11-05-90	B/H/O	0.4
	6019	f	01-29-92	D/D/D	0.1		4584	f	11-05-90	B/H/O	0.4
	6060	ms/ts	02-20-92	D^1/F/D+E	0.2						
	6073	f	02-27-92	D^2/F/D	0.2	Q	4835	ms/ts	02-12-91	M/K/D	0.2
		fluconazole	03-02-92				4961	f	03-13-91	M/K/D	0.2
	6424	ms/ts	06-22-92	D^3/G/D	0.2		4988	ts	03-25-91	M/K/D	0.2
	6611	f	07-29-92	D^4/H/F	0.4		5019	ms/ts	03-28-91	M/K/D	0.2
								fluconazole	03-28-91		
E	3212	ms/ts	02-06-90	E/C/A	0.4						
						R	5116	f	05-15-91	N/Q/A	0.2
F	4484	f	10-08-90	C/I/G	0.2		5117	ms/ts	05-15-91	N/Q/A	0.2
	4485	ms/ts	10-08-90	C/I/G	0.4		5161	ms/ts	05-31-91	N/Q/A	0.2
		fluconazole	10-08-90				5157	ms/ts	05-27-91	N/Q/A	0.2
	4517	S	10-19-90	C/I/G	0.4			fluconazole	05-27-91		
	4591	ms/ts	11-05-90	C/I/G	0.4		5200	ms/ts	06-16-91	N/Q/A	0.2
	4592	f	11-05-90	C/I/G	0.4						
						S	5252	f	06-27-91	O/R/P	0.2
							5367	ms/ts	07-25-91	O/R/P	-
G	6168	ms/ts	04-06-92	G/C/A	0.4		5473	ms/ts	08-24-91	O/R/P	0.2

	6319	ms/ts	05-21-92	H/I/I	0.4
		fluconazole	05-21-92		
	6713	ms/ts	08-17-92	H/I/I	100
H	3096	f	14-01-90	I/K/J	0.4
	3097	S	01-09-90	I/K/J	0.4
	3231	ms/ts	02-12-90	I/K/J	0.4
	3250	f	02-12-90	I/K/J	0.1
	3288	ms/ts	02-21-90	I/K/J	0.4
		fluconazole	02-23-90		
I	6339	ms	05-20-92	J/L/D	0.2
	6342	f	05-20-92	J/L/D	0.2
	6637	f	08-04-92	J/L/D	0.2
	6722	ms	08-19-92	J/L/K	0.2
		fluconazole	08-24-92		
	6840	ms/ts	09-11-92	J/L/K	1.6
J	6697	ms/ts	08-13-92	K'/C/A	0.2
		fluconazole	08-16-92		
	6711	f	08-17-92	K/M/A	0.2
	6735	ms/ts	08-24-92	K/M/A	0.2
K	6443	ms/ts	06-23-92	A'/C/L	0.2
	6453	f	06-23-92	L/N/A	0.2
	6512	ms	07-08-92	L/N/A	0.2
		fluconazole	07-08-92		
	6569	f	07-20-92	L/N/A	0.2
	6761	ms/ts	08-27-92	L/N/A	0.2
	6760	f	08-27-92	L/N/A	0.2
	6817	ms/ts	09-08-92	L/N/A	0.2
L	5343	ms/ts	07-22-91	C/O/M	0.2
		fluconazole	07-31-91		
	5391	ms/ts	08-01-91	C/O/M	0.2
M	5418	ms/ts	08-08-91	A/N/A	0.2
	5520	ms/ts	09-02-91	A/N/N	0.2
		fluconazole	10-03-91		
	5705	f	10-22-91	C/N/A	0.2
	5475	f	08-24-91	O/R/P	0.2
	5906	ms/ts	12-28-91	O/R/P	0.2
	5907	f	12-28-91	O/R/P	0.2
		fluconazole	12-30-91		
	6022	ms/ts	02-03-92	O/R/P	0.4
T	5464	ms/ts	08-26-91	A'/ND/A	0.4
	5521	ms/ts	09-02-91	A'/ND/A	0.4
	6152	ms/ts	03-23-92	A'/ND/A	0.2
	6169	ms/ts	04-02-92	A'/ND/A	0.2
	6186	f	04-09-92	A'/ND/A	0.2
		fluconazole	04-09-92		
	6225	ms/ts	04-27-92	A'/ND/A	0.2
	6240	ms/ts	05-04-92	A'/ND/A	0.2
		fluconazole	05-07-92		
	6270	ms/ts	05-11-92	A'/ND/A	0.2
U	5555*	f	09-13-91	P/ND/A	0.4
		fluconazole	10-06-91		
	6940*	ms/ts	10-05-92	Q/ND/Q	3.1
V	5951	f	01-10-92	R/ND/O	0.2
	5952	ts	01-10-92	R/ND/O	0.2
		fluconazole	01-22-92		
	6007	f	01-27-92	R/ND/O	3.1
W	5879	ms/ts	12-12-91	A'/ND/D	0.2
	5881	f	12-12-91	A'/ND/D	0.2
	6402	ms/ts	06-15-92	A/ND/D	0.4
	6483	ms/ts	07-07-92	A/ND/D	0.2
		fluconazole	07-09-92		
	6559	f	07-16-92	A'/ND/A	0.2
	6704	ms	08-04-92	S/ND/A	0.2
	6734	b	08-07-92	A''/ND/D	0.2
X	6191	f	04-08-92	T/ND/ND	0.4
	6192	ms/ts	04-08-92	T/ND/ND	0.4
		fluconazole	04-23-92		
	6243	ms/ts	05-04-92	T/ND/ND	0.4

Note: P=patient; ND=not determined; MS=mouth swab; MIC=minimal inhibitory concentration; TS=throat swab; f=fecal isolate; s=sputum; b=blood isolate; I: primer combination 1245+1246; II: primer combination; 1236+1245; III: primer 1251. Genotypic codes D' to D^4 represent slightly different DNA banding patterns. Codes accompanied by an ' are strongly resembling the pattern type without the hyphen. The starting date of fluconazole treatment is indicated. *Upon retyping it became apparent that these isolates do not represent *C. albicans*. Isolate 5555 was shown to be a *C. kefir* strain, whereas isolate 6940 appeared to belong to the species *C. glabrata*. Note that the fingerprints for these isolates are unique.

Table 2 Primers used for interrepeat PCR typing of *Candida albicans*

code	nucleotide sequence	code	ref.
	prokaryotic repeat motifs		
1236	TACATTTCGAGGACCCCTAAGTG	BGO2	30
1245	AAGTAAGTGACTGGGGTGAGCG	ERIC2	33
1246	ATGTAAGCTCCTGGGGATTCAC	ERIC1	33
	eukaryotic repeat motifs		
1198	GGAGGAGGAGGAGGAGGAGGA	(GGA)7	15, 31
1251	TGGGTGTGTGGGTGTGTGGGTGTG	TELO1	15, 31
1262	TAGGGTAGGGTAGGGTAGG	TELO2	15

Note: ERIC = enterobacterial repetitive intergenic consensus;
TELO = telomeric repeat motif.

RESULTS

PCR genotyping of *C. albicans* reference strains: Results of PCR genotyping using prokaryotic consensus repeat motifs and eukaryotic repeat-like primers are shown in Figure 1. The right part of Table 3 surveys the interpretation of the banding patterns, which are encoded by letters (A to G). The number of identifiable, unique PCR banding patterns per assay varies from three to seven. When all banding patterns were combined to an 8-digit code, 13 different types could be discriminated in 21 isolates. It has to be emphasised that not all PCR tests displayed in Figure 1 contributed equally to the detection of interstrain variation. If the results of the PCRs guided by primers 1245/1246 and primer 1251 are combined, also 13 different strains can be identified (see below).

For 20 of the *C. albicans* reference strains, several other typing assays have been used by others (Table 3) (13, 25). These procedures discriminate between 8 and 12 genetically different strains, respectively. This implies that the resolution of PCR genotyping is in the same order of magnitude as that of the other procedures. The results of all typing procedures show a certain degree of overlap, which underscores the reliability of PCR genotyping. Isolates 711-713 could not be discriminated by PCR amplification, HinfI RFLP or PFGE chromosome typing. Isolates 714 and 717 were identical by PCR genotyping, HinfI RFLP and EcoRI RFLP. Several other examples are obvious from Table 3.

Figure 1 reveals that the quality of the banding patterns varied with primer choice. For the eukaryotic repeat consensus primers, the results obtained with the combinations rather than with single primers were distinct.

Figure 1A. Polymerase chain reaction (PCR) genotyping of DNA isolated from reference panel of *C. albicans* strains (table 3): interrepeat PCR assays. Lane numbers are strain numbers in table 3. Lane M contains length markers (1-kb ladder); lower and upper arrows in each panel indicate 500 and 1600 bp fragments, respectively. Numbers at left correspond to primer numbers in Table 2. A. Prokaryotic repeat consensus primers in low stringency of annealing (25°C) PCR test.

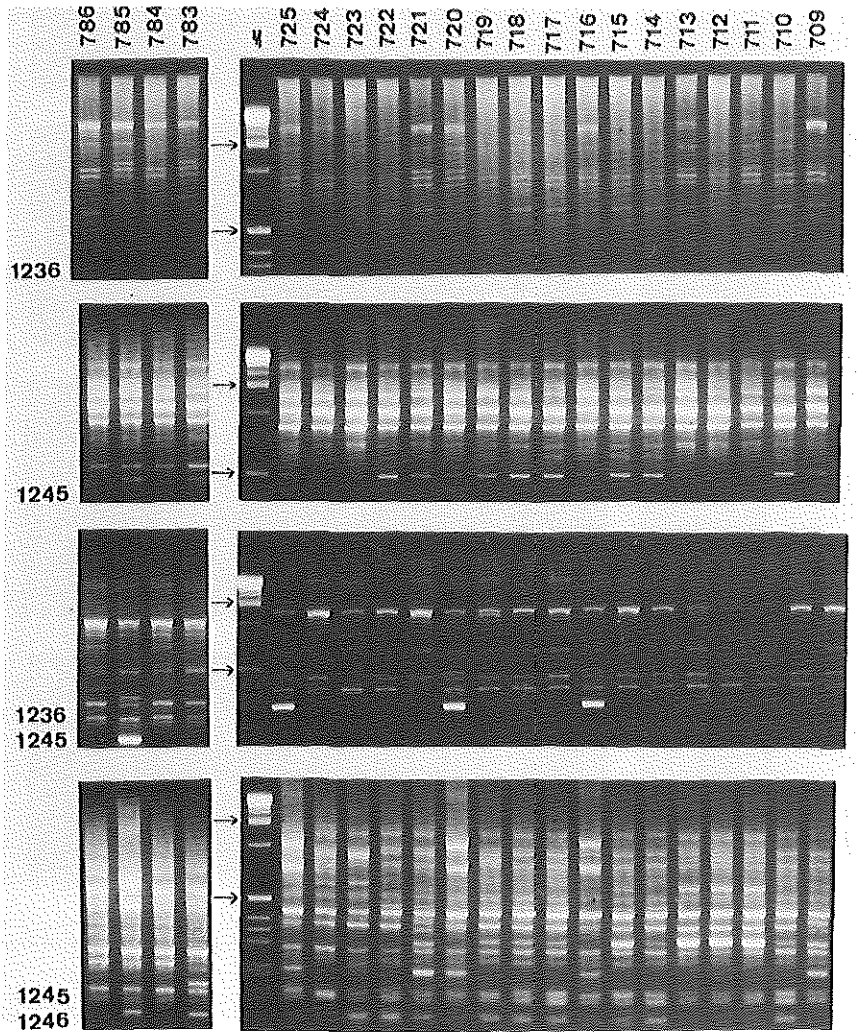


Figure 1B. Eukaryotic repeat consensus primers in high stringency of annealing PCR (52°C) test.

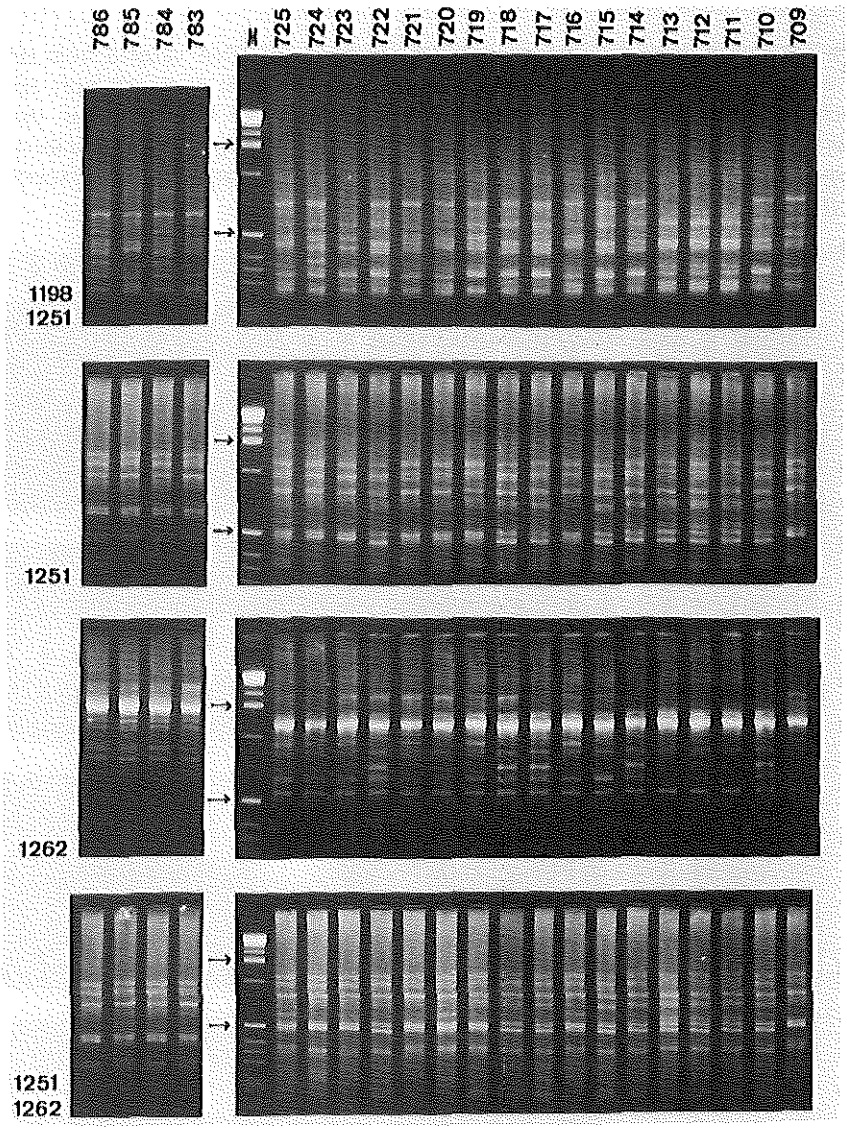


TABLE 3 SURVEY OF GENOTYPING STUDIES PERFORMED ON THE *C. ALBICANS* REFERENCE PANEL

	GENERAL GENOTYPING ASSAYS					PCR ASSAYS								
	EcoRI	27a	PFGE	HinfI type	major band HinfI RFLP	1236 I	1245 II	1236/ 1245 III	1245/ 1246 IV	1251/ 1198 V	1251 VI	1262 VII	1262/ 1251 VIII	overall PCR type
709	IB3	1	C1	H1	h1	A	A	A	A	A	A	A	A	1
710	IB3	2	C2	H2	h2	B	B	B	B	B	B	B	B	2
711	IA2	3	C2	H3	h3	A	B	C	C	C	C	C	C	3
712	IA2	4	C2	H4	h3	A	B	C	C	C	C	C	C	3
713	IA2	3	C2	H5	h3	A	B	C	C	C	C	C	C	3
714	IA8	2	C3	H2	h2	B	B	B	B	B	B	B	B	2
715	IA8	5	C4	H7	h2	C	A	B	B	B	D	A	B	4
716	IA11	6	C5	H8	h4	A	C	D	D	D	E	A	D	5
717	IA8	7	C4	H2	h2	B	B	B	B	B	B	B	B	2
718	IA8	8	C6	H7	h2	C	C	B	B	B	B	B	B	6
719	IA12	9	C7	H9	h6	B	B	C	B	A	E	A	D	7
720	IA11	6	C5	H9	h6	A	C	D	D	D	E	A	D	5
721	IA3	10	C9	H10	h7	A	A	A	A	A	E	A	D	8
722	IA8	8	C11	H12	h8	B	C	B	E	B	B	B	C	9
723	1B4	12	C2	H13	h9	B	A	E	F	E	A	A	A	10
724	IA12	11	C2	H11	h6	D	D	A	G	F	A	A	A	11
725	IA11	10	C5	H9	h6	A	C	D	D	D	E	A	D	5
784	IA12	11	C2	H11	h6	D	D	A	B	F	A	A	A	12
785	IA11	6	C8	H9	h6	A	C	D	D	D	E	A	D	5
786	IA12	11	C10	H11	h6	D	D	A	E	F	A	A	A	13
783	ND	ND	ND	ND	ND	A	A	A	A	A	E	A	D	8

Note: The results described in column 2 to 6 summarize those presented in references 6 and 14. The column marked 27a displays the results of hybridization of Southern blots with a repeat specific DNA probe, PFGE represents karyotyping with the aid of pulsed field gel electrophoresis. The columns 2, 5 and 6 (marked "EcoRI", "HinfI type" and "major band HinfI RFLP") show data obtained by restriction fragment polymorphism analysis. The eight additional columns summarize the fingerprinting assays presented in Figure 1A and 1B, according to the specific primer combination. The column on the right combines all PCR results in a single genotypic code.

primers were distinct. The 1236/1245 combination rendered clear and discrete banding patterns, which appeared to be somewhat less discriminatory than the 1245/1246 combination (5 vs. 7 identifiable patterns, respectively). With eukaryotic repeat primers, a similar phenomenon is noticed. In this case, the assays involving primer 1251 most clearly determined a notable degree of genetic variation: primer 1251 is most effective for the detection of DNA polymorphism in *C. albicans*. Addition of primer 1262 in the same PCR did not add to the detection of DNA variation. On the contrary, it reduced the number of variants from 5 to 4.

In summary, adequate discrimination of *C. albicans* strains can be attained, but combination of the results from multiple assays is required for maximum resolution. The assays are reproducible because new cultures of the same strain rendered identical genotypes. When multiple colonies (n=25) of a single strain or isolate (n=10) were analyzed, all DNA preparations again rendered identical and reproducible PCR fingerprints.

PCR genotyping of clinical *C. albicans* isolates: Twenty-four neutropenic patients were regularly screened for *C. albicans* colonization. A selection of 117 isolates (1-10 per patient) was examined with the three most discriminating PCR assays. Assays I and II (Table 1) involved prokaryotic repeat motif primers, whereas the third PCR assay was performed with the yeast telomer specific primer TELO 1. Results of PCR with 1236/1245 did not add to the variability already determined by the other two assays. This observation is in concordance with that noted for the reference panel of *C. albicans* strains, and this is also the reason why the assays with 1245/1246 and 1251 were selected for screening of clinical isolates. All patterns obtained, a selection of which is shown in Figure 2, were again identified by an uppercase letter (Table 3), and the results are presented in Table 1. Table 1 also shows the dates of the start of fluconazole treatment.

Most patients remained colonised with the same *C. albicans* strain for the entire study period, irrespective of fluconazole treatment. These patients were A-C, H, L, N, O, Q to T, V and X, constituting the majority of the study population. Since for patient E only a single isolate was available, it will not be discussed further.

In the other patients, a certain degree of genotypic variation in the colonizing strain(s) is observed. Patient D was apparently colonised by a mixture of closely related strains, both before and after fluconazole treatment. Patient W displayed a similar form of variation in colonization. In four patients, on a single occasion, a deviating strain was isolated. For all 4 patients (G, I, K and P), this occurred before fluconazole treatment. In patient J, an apparent switch in colonization was observed. Initially, genotype JLD was found. Later, that strain was replaced by an isolate of genotype JLK. The strains share a certain degree of homology, as two of three PCR assays showed identical results. This form of recolonization (if so) is not associated with fluconazole treatment (Table 1). Patients M displayed a genotypic change in the *C. albicans* strain, and patient U had two non *C. albicans* strains (in faeces and the oral cavity).

Comparisons of PCR genotypes: When the results displayed in Figure 1 and Figure 2 are compared, some striking differences and similarities are observed. When primer 1251 was applied, both the American reference strains and the Dutch clinical isolates displayed similar DNA genotypes. Also, the degree of variation appears to be similar. In all cases, a prominent doublet or triplet of fragments of about 500 bp long was found, whereas the other DNA fragments generated by the PCR did not exceed an approximate length of 1200 bp. All electrophoretic lanes had a rather high degree of background staining, which could indicate asymmetric PCR (unpublished data). In the results obtained with primers 1245/1246, this similarity is less obvious. Although a common band, observed near the 450 bp length marker, was clear in some cases (patients P, S and T) this did not apply to all clinical isolates.

On superficial inspection, our clinical isolates could be divided into two major groups. Patients B-F, H, K, L, N-Q, and S-Y harboured strains whose 1245/1246 PCR amplified DNA fragments were in the 200 bp region. In patients A, J, I, G, M, and R another basic pattern arose. Apparently, two major groups of *C. albicans* strains can be discerned.

Susceptibility studies: MICs of fluconazole ranged from <0.05 to 100 μg/ml. All but one isolate remained susceptible to fluconazole during treatment. The resistant isolate (MIC, 100 μg/ml) had the same genotype as the susceptible strain (MIC, 0.4 μg/ml) isolated before fluconazole treatment.

DISCUSSION

Advantages of PCR-mediated amplification of fungal DNA for the determination of genetic characteristics are speed and simplicity. This allows discrimination of an ongoing epidemic from a coincidental increase in sporadic infections within a working day. Previously, the latter could be achieved only by time-consuming, technically complex procedures (19, 21, 23). Also, for PCR genotyping, DNA need not be of very high quality, as in karyotyping studies. As long as the average length of DNA fragments exceeds 10 kb, reproducible patterns are generated. However, the accepted molecular procedures used to determine genetic similarity in *C. albicans* are effective and reproducible (18, 26, 27, 36). Therefore, PCR-mediated procedures must first be compared with these typing methods. For this reason, we initially characterised a set of well-studied *C. albicans* reference strains by PCR, after which PCR genotyping was applied for genetic characterization of clinical isolates.

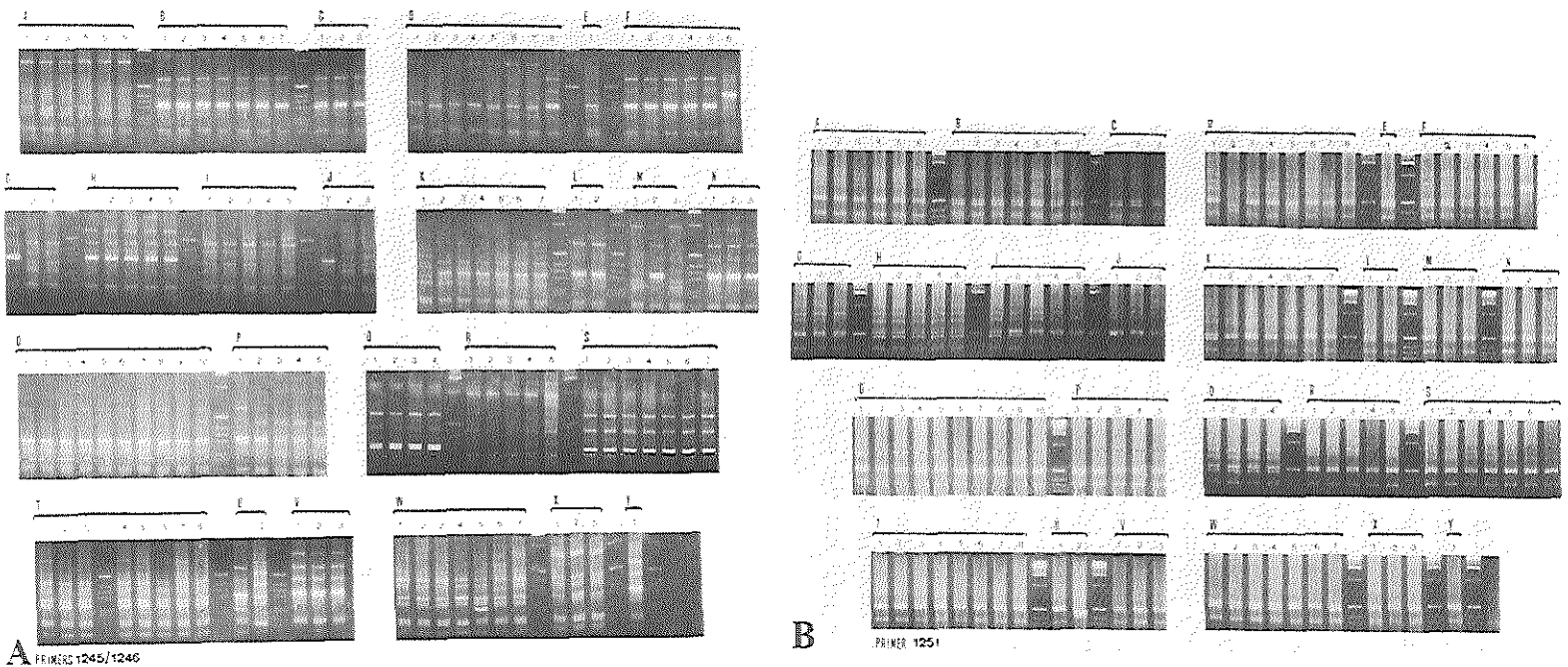


Figure 2 Polymerase chain reaction (PCR) genotyping of DNA isolated from serial *C. albicans* strains obtained from neutropenic patients: interrepeat PCR assays. Letters are patients and numbers are isolates, as in Table 1. Length marker (unnumbered lanes) is same as shown in Figure 1. Rightmost lane in WXY panels shows negative control samples (no DNA added before PCR). **A.** Prokaryotic repeat consensus primers 1245/1246. **B.** Eukaryotic telomeric repeat motif primer 1251. Isolate from patient Y is not included in clinical discussion, nor has its fluconazole sensitivity been determined.

Discrimination of individual *C. albicans* strains: Among the reference strains, 13 types could be identified by combining EcoRI RFLP and 27A probe-mediated DNA genotyping (13). In our study, the same resolution is achieved by application of two PCR assays. PCR confirmed some of the close relationships defined by other procedures, but also discriminated otherwise identical strains. Proven examples of closely related strains are 711 and 713, 716 and 720 (Table 3). Strains 724 and 784 are examples that can be discriminated by PCR with 1245/1246, but not by the other typing assays. Of note, in the collection of 20 isolates by a combination of the results of all procedures, 20 genetically different yeast strains are discernable.

Typing of clinical *C. albicans* isolates: In two recent reports (26, 27), DNA typing with the repetitive sequence Ca3 was used to identify *C. albicans* strains isolated from diverse patient populations. In those studies, geographic clustering of *C. albicans* types seemed to occur. Persistence of the same strain in recurrent vaginitis has been demonstrated (22, 29). Other typing data reveal that also in AIDS patients (18, 27, 36) and in others (6, 34), the same strain of *C. albicans* persists. No clear discrimination between the average strain and strains particularly found in AIDS patients can be made (26, 34). In the present study, persistence of a genotypically constant strain occurred in most neutropenic patients.

Exceptions are found, possibly indicating multiple colonization, but it must be emphasised that in most of these patients, only a single aberrant isolate was found. Two patients displayed a clear switch in colonizing strain. Since this occurred in only 2 of 24 patients, the relevance of the observation remains to be confirmed. No relation could be found with fluconazole use. In only one patient were clinical treatment failure and in vitro resistance to fluconazole observed in a strain of constant genotypic characteristics. The low incidence of fluconazole-resistant *C. albicans* after a few courses (one to three) of the drug compares with previous findings in AIDS patients. However, in those patients, the long-term use of fluconazole (up to 600 days) as suppression therapy for oral thrush or oesophageal candidiasis has been associated with incidental reports of the development of resistant strains (5, 7, 9, 11, 14, 20, 37). Whether these resistant strains are genotypically similar to pre-treatment strains or new colonizers was not reported.

There are several known mechanisms of azole resistance in *Candida* species, including decreased azole susceptibility of p-450-dependent 14-α-sterol methylase, reduced azole membrane permeability, and, more speculative, an overproduction of p-450 (32). In our hands molecular typing methods are at present inadequate to predict the development of fluconazole resistance. A similar conclusion was reached in a genotyping study of *C. albicans* isolates from AIDS patients (1).

Because of the diversity in *C. albicans* types in individual patients, nosocomial transmission of yeast strains is highly unlikely. This indicates efficient hospital hygiene systems and adequate patient isolation. All colonies directly isolated from clinical samples can be typed by PCR and this analysis can be done prospectively and longitudinally.

Additional studies will eventually lead to more insight into the dynamics of *C. albicans* colonization in any patient. Calibration of this procedure will also allow the in vivo evaluation of effects that treatment with fungistatic drugs may have on the *C. albicans* populations existing in healthy or diseased persons. Since the change in resistance phenotype was not accompanied by major genetic alteration, serial isolates of genetically comparable strains may allow cloning of resistance genes by differential RNA analyses (35). In conclusion, we found that PCR amplification of variable DNA domains allows typing of *C. albicans* strains. The use of this procedure allows both patient monitoring and studies of *C. albicans* variation and clonality.

REFERENCES

1. **Bart-Delabesse, E., P. Boiron, A. Carlotti, and B. Dupont** (1993) *Candida albicans* genotyping in studies with patients with AIDS developing resistance to fluconazole. J. Clin. Microbiol. **31**: 2933-2937.
2. **Boom, R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-Van Dillen, and J. van der Noordaa** (1990) Rapid and simple method for purification of nucleic acids. J Clin Microbiol **28**: 495-503.
3. **Caugant, D.A., and P. Sandven** (1993) Epidemiological analysis of *Candida albicans* strains by multilocus enzyme electrophoresis. J. Clin. Microbiol. **31**: 2-15-220.
4. **De Bruyn, F** (1992) The use of repetitive (Repetitive Extragenic Palinchromic and Enterobacterial Repetitive Intergenic Consensus) sequences and the PCR to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58**: 2180-2187.
5. **Fan-Havard, P., D. Capano, S.M. Smith, A. Mangia, and R.H.K. Eng** (1991) Development of resistance in *Candida* isolates from patients receiving prolonged antifungal therapy. Antimicrob. Agents Chemother. **35**: 2302-2305.
6. **Fox, B.C., H.L. Hobley, and J.C. Wade** (1989) The use of a DNA probe for epidemiological studies of candidiasis in immunocompromised hosts. J. Infect. Dis. **159**: 488-494.
7. **Fox, R., K.R. Neal, C.L. Leen, M.E. Ellis, and B.K. Mandal** (1991) Fluconazole resistant Candida in AIDS. J. Infect. **22**: 201-204.
8. **Fraser, V.F., M. Jones, J. Dunkel, S. Starfer, G. Medoff, and W.C. Dunagan** (1992) Candidemia in a tertiary care hospital: epidemiology, risk factors and predictors of mortality. Clin. Infect. Dis. **15**: 414-421.
9. **Jangrineti, A., J.K. Carmichael, and K. Campbell** (1993) Fluconazole-resistant *Candida albicans* after long-term suppressive therapy. Arch. Intern. Med. **153**: 1122-1124.
10. **Khattak, M.N., J.P. Burnie, R.C. Matthews, and B.A. Oppenheim** (1992) Clamped homogeneous electric field gel electrophoresis typing of *Torulopsis glabrata* isolates causing nosocomial infections. J. Clin. Microbiol. **30**: 2211-2215.
11. **Kitchen, V.S., M. Savage, and J.R.W. Harris** (1991) *Candida albicans* resistance in AIDS. J. Infect. **22**: 204-205.

12. **Lehmann, P.F., D. Lin, and B.A. Lasker** (1992) Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. J. Clin. Microbiol. **30**: 3249-3254.
13. **Magee, P.T., L. Bowdin, and J. Staudinger** (1992) Comparison of molecular typing methods for *Candida albicans*. J. Clin. Microbiol. **30**: 2674-2679.
14. **Ng, T.T.C., and D.W. Denning** (1993) Fluconazole resistance in *Candida* in patients with AIDS - a therapeutic approach. J. Infect. **26**: 117-125.
15. **Niesters, H.G.M., W.H.F. Goessens, J.F.G.M. Meis, and W.G.V. Quint** (1993) Rapid polymerase chain reaction based identification assays for *Candida* species. J. Clin. Microbiol. **31**: 904-910.
16. **Odds, F.C., D.L. Brawner, J. Staudinger, P.T. Magee, and D.R. Soll** (1992) Typing of *Candida albicans* strains. J. Med. Vet. Mycol. **30, suppl. 1**: 87-94.
17. **Odds, F.C., J. Schmid, and D.R. Soll** (1990) Epidemiology of *Candida* infections in AIDS. In: Van den Bossche H et al. Mycoses in AIDS patients. New York: Plenum Press, 67-74.
18. **Powderly, W.G., K. Robinson, and E.J. Keath** (1992) Molecular typing of Candida albicans isolated from oral lesions of HIV-infected individuals. AIDS **6**: 81-84.
19. **Reagan, D.R., M.A. Pfaller, R.J. Hollis, and R.P. Wenzel** (1990) Characterization of the sequence of colonization and nosocomial candidemia using DNA fingerprinting and a DNA probe. J. Clin. Microbiol. **28**: 2733-2738.
20. **Redding, S., J. Smith, G. Farinicci, M.A. Pfaller, M. Rinaldi, and A. Fothergill** (1993) Development of *Candida albicans* resistance to fluconazole during treatment for thrush over a two-year period in AIDS. In: Program and Abstract of the 93rd ASM General Meeting. New Orleans, number 527.
21. **Rustchenko, E.P.** (1991) Variations of *Candida albicans* electrophoretic karyotypes. J. Bacteriol. **173**: 6586-6596.
22. **Sadhu, C., M.J. McEachern, E.P. Rustchenko, J. Schmid, D.R. Soll, and J.B. Hicks** (1991) Telomeric and dispersed reat sequences in *Candida* yeasts and their use in strain identification. J. Bacteriol. **173**: 842-850.
23. **Sanchez, V., J.A. Vazquez, D. Barth-Jones, L. Dembry, J.D. Sobel, and M.J. Zervos** (1992) Epidemiology of nosocomial acquisition of *Candida lusitaniae*. J. Clin. Microbiol. **30**: 3005-3008.
24. **Saral, R** (1991) *Candida* and *Aspergillus* infections in immunocompromised patients: an overview. Rev. Infect. Dis. **13**: 487-492.
25. **Scherer, S., and D.A. Stevens** (1987) Application of DNA typing methods to epidemiology and taxonomy of *Candida* species. J. Clin. Microbiol. **25**: 675-679.
26. **Schmid, J., M. Rotman, B. Reed, C.L. Pierson, and D.R. Soll** (1993) Genetic similarity of *Candida albicans* strains from vaginitis patients and their partners. J. Clin. Microbiol. **31**: 39-46.
27. **Schmid, J., F.C. Odds, M.J. Wiselka, K.G. Nicholson, and D.R. Soll** (1992) Genetic similarity and maintenance of *Candida albicans* strains from a group of AIDS patients, demonstrated by DNA fingerprinting. J. Clin. Microbiol. **30**: 9-35-941.
28. **Shawar, R., V. Paetznich, Z. Witte, L. Gamsey Ensign, E. Anaissie, and M. LaRocco** (1992) Collaborative investigation of broth microdilution and semisolid

agar dilution for in vitro susceptibility testing of *Candida albicans*. J. Clin. Microbiol. **30**: 1976-1986.

29. **Soll, D.R., R. Galask, and S. Isley** (1989) Switching of *Candida albicans* during successive episodes of recurrent vaginitis. J. Clin. Microbiol. **27**: 681-690.
30. **Van Belkum, A., R. Bax, P. Peerbooms, W.H.F. Goessens, N. van Leeuwen, and W.G.V. Quint** (1993) Comparison of phage typing and DNA fingerprinting by PCR for discrimination of methicillin resistant *Staphylococcus aureus* strains. J Clin Microbiol **31**: 798-803.
31. **Van Belkum, A., J. de Jonckheere, and W.G.V. Quint** (1992) Genotyping *Naegleria* species and *Naegleria fowleri* isolates by interrepeat polymerase chain reaction. J. Clin. Microbiol. **30**: 2595-2598.
32. **Vanden Bossche, H., P. Marichal, F.C. Odds, L. Le Jeune, and M.C. Coene** (1992) Characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob. Agents Chemother. **36**: 2602-2610.
33. **Versalovic, J., T. Koeuth, and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19**: 6823-6831.
34. **Wade, J.C** (1993) Epidemiology of Candida infections. In: Bodey GP, ed. Candidiasis: Pathogenesis, diagnostics and treatment. 2nd ed. New York: Raven Press, pp. 85-107.
35. **Welsh, J., K. Chada, S.S. Dalal, R. Cheng, D. Ralph, and M.M. McClelland** (1992) Arbitrary primed PCR fingerprinting of RNA. Nucleic Acids Res. **20**: 4965-4970.
36. **Whelen, W.L., D.R. Kirsch, K.J. Kwon-Chung, S.M. Wahl, and P.D. Smith** (1990) *Candida albicans* in patients with the acquired immunodeficiency syndrome: absence of a novel or hypervirulent strain. J. Infect. Dis. **162**: 513-518.
37. **Willochs, L., C.L.S. Leen, R.P. Brettle P, T.B. Urquhart Russell, L.J.R. Milne** (1991) Fluconazole resistance in AIDS patients. J. Antimicrob. Chemother. **28**: 937-939.

Adapted from: Bone Marrow Transpl. 1994;13:811-815

CHAPTER VII

PCR MEDIATED GENOTYPING OF *CANDIDA ALBICANS* STRAINS FROM BONE MARROW TRANSPLANTATION PATIENTS

Alex van Belkum, Wendy Mol, Rik van Saene[1], Lynne M. Ball[1], Dick van Velzen[1] and Wim G.V. Quint[2]

[1]Royal Childrens Hospital "Alder Hey", Liverpool, United Kingdom. [2]Diagnostic Centre SSDZ, Department of Molecular Biology, P.O. Box 5010, 2600 GA Delft, The Netherlands.

SUMMARY

DNA amplification using polymerase chain reaction (PCR) primers aiming at eukaryotic or prokaryotic repetitive DNA motifs enables discrimination between individual *Candida albicans* isolates. This PCR mediated DNA fingerprinting procedure was used to monitor yeast colonisation in immune-compromised leukemia patients (n=11), who were undergoing bone marrow transplantation (BMT). The leukaemia patients remained colonised by the same strain throughout a five month study period, irrespective of intermediate treatment with fungostatics or application of BMT-related therapies. All 11 strains could be identified separately by PCR fingerprinting. This implies that spreading of *C. albicans* from patient to patient does not seem to occur in this study group, despite the fact that medical employees frequently travel between wards which are in close proximity to other departments harbouring neutropenic patients. Several of these patients (n=35) were also monitored for *C. albicans* colonisation and strain typing corroborated the lack of extensive cross-contamination during the study period. The application of PCR-mediated genotyping of fungi in epidemiological analyses and evaluation of hospital hygiene is discussed.

INTRODUCTION

Prevention of cross-contamination by infectious agents between patients within a single hospital is of major medical concern (8, 17). However, despite the application of several stringent control measures, nosocomial epidemics cannot always be accurately prevented (2). The risk for endemic outbreaks involving pathogenic micro-organisms is greatest where large groups of immunocompromised individuals are treated in close proximity. These groups vary from newborn infants (18) to AIDS or leukaemic patients (1, 19). Examples of lifethreatening combinations are AIDS patients infected with (multiresistant) *Mycobacterium tuberculosis* (4), neonates presenting with fulminant *Streptococcus pyogenes* infections (3) or haemato-oncologic patients with systemic fungal infections (22). In order to prevent extensive fungal dissemination in this latter class of patients, strict surveillance measures are usually taken. To evaluate the efficiency of these methods, screening procedures enabling direct comparison of fungal isolates deriving from various patients nursed in different but neighbouring wards must be available (15).

By comparative analyses, potential sources of infection can be traced and, within individual patients, identification of colonising strains can be performed. Spreading of micro-organisms can also be studied accurately (16).

For typing of microorganisms in such a way that different isolates of a single species can be discriminated, several molecular biological procedures have been developed and applied in a large number of epidemiological studies (14, 15, 16, 21). Restriction fragment length polymorphism (RFLP), detection of which can take place by various electrophoretic strategies, provides a useful means of identifying and typing genomes of diverse microbes (5). Another DNA typing method involves the amplification of loci which are polymorphic among closely related organisms, by the polymerase chain reaction (PCR) (9, 28, 29). By an appropriate preselection of the type of region, efficient PCR identification tests can be designed. The DNA of vertebrates, for instance, harbours a multitude of repetitive DNA sequences displaying extensive allelic variation (27). These loci can be used for digitised molecular typing assays (10). The bacterial homologues of these repeat motifs are the repetitive extragenic palindromes (REP) and the enterobacterial repetitive extragenic consensus (ERIC) sequences (26). Although different in nature, these sequences harbour several characteristics which are comparable to those of the eukaryotic repeat sequences. Amongst these, are the variability and their putative applicability in PCR-mediated genotyping studies (7, 23).

In the present study, a PCR fingerprinting procedure, aiming at the amplification of repetitive, variable DNA domains present in the genome of *Candida albicans* is applied to longitudinally monitor yeast colonisation in severely immunocompromised patients selected for bone marrow transplantation (BMT) or hospitalised for other forms of cancer treatment. Individual patients were regularly screened for the presence of *C. albicans* and the collected strains were stored and genotyped by PCR. Also, yeast isolates from patients nursed in neighbouring wards were included in the study. Technical procedures and epidemiological implications will be discussed.

MATERIALS AND METHODS

Patient population: Patients eligible for (autologous) BMT (n=11) were included in a 12-13 week treatment program. The regimen included treatment with methotrexate (MTX), both oral and intrathecal if required by protocol. Patients received asparaginase (6000 U/m^{20U}),vincristine (1.5 mg/m^2), daunorubicine (45 mg/m^2), prednisolone (40 mg/m^2), 6-mercaptopurine (75 mg/m^2) and cotrimoxazole. Between weeks 6 and 8 cranial gamma-irradiation (18 Gy) was given. In some cases, VP16 (100 mg/m^2 iv), cytosine arabinoside (Ara C, 100 mg/m^2, iv, 12 hourly) and thioguanine (80 mg/m^2 po) were administered during 5 days in the fifth week of treatment. The age of the eleven BMT patients varied from 1 to 11 years (mean value of 5.2 years). All patients were seriously immunocompromised and suffered from *Candida* infection. They were all treated in a single BMT unit. The unit consisted of four cubicles, adjacent to each other and integra-

ted in the haematology/oncology ward of the Royal Children's Hospital "Alder Hey" in Liverpool. From this ward, other patients also suffering from *C. albicans* infection (n=35), were selected during the same period that the BMT patients were present. These paediatric patients (aged 5 months to 12 years) were hospitalised for pre-induction, for a neutropenic septicemic period or for chemotherapy against solid tumours. For most patients both rectal and throat swabs were obtained. Additional sites were sometimes sampled, including several types of (respiratory) tubing. When systemic yeast infection originating from the respiratory tract was diagnosed, patients were treated with cotrimoxazol or liposomal amphotericin B as prescribed by the manufacturer of these fungostatic compounds. A survey of patient data is presented in Figure 1.

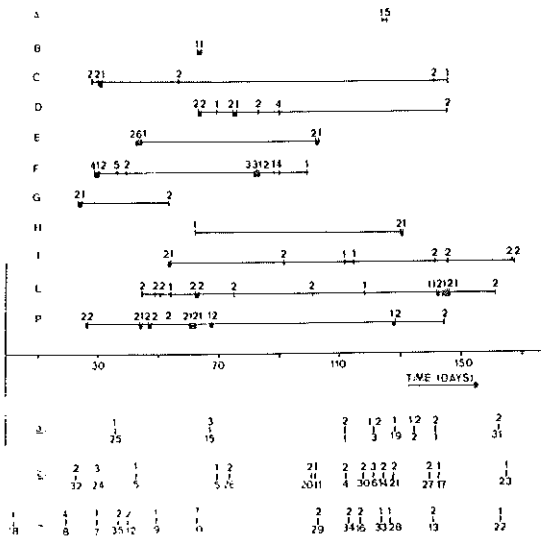


Figure 1 The 11 BMT patients (A through P) are indicated above the horizontal bar, which displays the *C. albicans* monitoring period for these various children. The small vertical lines indicate sampling dates, the anatomical origin is given by arabic numbering (1 = rectal swab, 2 = throat swab, 3 = nasogastric aspirate, 4 = nasopharyngeal aspirate, 5 = extra tracheal aspirate, 6 = sputum sample, 7 = sample from central venous line). Below the time bar, sampling dates for the haemato-oncology patients (numbered 1 to 35) are indicated by small vertical lines. Anatomical sites are numbered as above, the affixes indicate the reason for hospitalisation. (a. = neutropenic septicemic period; b. = chemotherapy for solid tumor; c. = pre-induction).

***C. albicans* isolation and storage:** Yeast cultures were identified using standardised microbiological procedures. *C. albicans* isolates were stored at 4°C on the original Sabouroud's agar plates. The plates were processed batchwise once a month. In general, a single colony was suspended in 2-3 ml 0.9% NaCl in water and the suspension was stored at 4°C until further use. Altogether 123 strains were isolated from the entire patient population. For five independent patients, multiple *C. albicans* colonies (n=25-50) were collected from the primary culture to examine the genetic homogeneity of the colonising isolate. From 30 patients from the haemato-oncology ward single isolates were obtained,

from the other 16 patients (including the BMT candidates) between 2 and 17 isolates were collected. The collection period varied from days up to four months (Figure 1).

DNA isolation: Samples of the stored *C. albicans* suspensions was plated on solid agar media. Plates were incubated at 37°C for about 60 h. When colony size was in the order of 4-5 mm in intersection, a single colony was picked and resuspended in 100 µl of a buffer containing 25 mM K_2HPO_4 pH 7.0, 1 M sorbitol, 2.5 mM ß-mercaptoethanol. To this suspension, 30 µl of a solution of Zymolyase (10 mg/ml, ICN Immunobiologicals, USA) was added and the mixture was incubated at 30°C for 1 h. The spheroplasts thus prepared were lysed by the addition of 1 ml of a buffer containing 4M guanidine-isothiocyanate and celite. Isolation of DNA was performed as described previously (6). DNA preparations were stored in a final volume of 200 µl in 10 mM Tris.HCl pH 8.0, 1 mM EDTA at -20°C.

PCR producedures: Amplification reactions were performed in 100 µl volumes consisting of 10 mM Tris.HCl pH 9.0, 50 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM deoxyribonucleotide triphosphates, 50 pmol of primer(s) and 0.5 units of Taq DNA polymerase (Super Taq, Sphaero Q, The Netherlands). Template DNA (10 µl) was added and 40 cycles of repeated denaturation, primer annealing and enzymatic chain extension was performed in a Biomed thermocycler (model 60). Primers 799 and 944 ($(GGA)_7$ and $(TGGGTGTG)_3$, respectively) require a program consisting of 40 rounds of 1 min 94°C, 2 min 52°C and 3 min 74°C. The BG2 and ERIC2 primers (5'-TA-CATTTCGAGGACCCCTAAAGTG-3' and 5'-AAGTAAGTGACTGGGGTGAGCG-3') were used in a program that alternates between 1 min 94°C, 1 min 25°C and 2 min 74°C. The latter program was preceded by incubation at 94°C for 5 min and, after 40 cycles, followed by a 10 min incubation at 74°C. The amplified DNA was electrophoresed through 1-2.5% agarose gels in 40 mM Tris.HAc pH 7.5, 1 mM EDTA. PCR fingerprints were visualised by ethidium bromide staining and recorded on Polaroid 767 photos.

RESULTS

Since PCR fingerprinting is a relatively new technique and since little is known about the genetic homogeneity of colonising or infecting *Candida* strains, an experiment aiming at the characterisation of the population structure of *C. albicans* was initially undertaken in a few patients. For five patients, 25-50 colonies were picked and DNA was isolated for all individual "clones". All yeast clones obtained from single patients were identical with respect to their PCR fingerprints (data not shown). Although not absolute proof for a clonal population structure, this result emphasizes the homogeneity of strains deriving from a single patient. It was assumed from this result that the selection of a single colony of the primary *C. albicans* culture does not lead to biased analyses (see also below).

Typing of longitudinal *C. albicans* isolates from BMT patients: For the BMT patients an overall number of 84 *C. albicans* isolates were collected and DNA fingerprin-

ting was performed using two to four different PCR primers or PCR primer combinations. For all patients the assays involving primer 799 and the combination of primers 799 and 944 were carried out. For patients A to E, G, H, I and P the BG2/ERIC2 primer combination was added. The ERIC2 test was performed for patients A to E and G to P. The overall number of PCRs amounted to 291. By giving the different DNA banding patterns various arabical indices, the genotype could be established as a 2 to 4 letter code. It appeared that in this group of 84 isolates from 11 patients 12 different *C. albicans* genotypes were identifiable. This implies that 10 of 11 patients remained colonised by the same strain during the entire study period and that all individual strains could be identified (100% resolution). In only one patient (P) was a deviating strain was encountered on a single occasion. Interestingly, this concerned a duplicate isolate, representing a strain isolated from the throat in the first candidemic period. Retrospectively, it could not be deduced whether the presence of this strain was due to contamination but in the light of first paragraph of this section it could be that coincidentally a clone was isolated belonging to a highly underrepresented genotype in the endogenous (mixture of) strain(s)

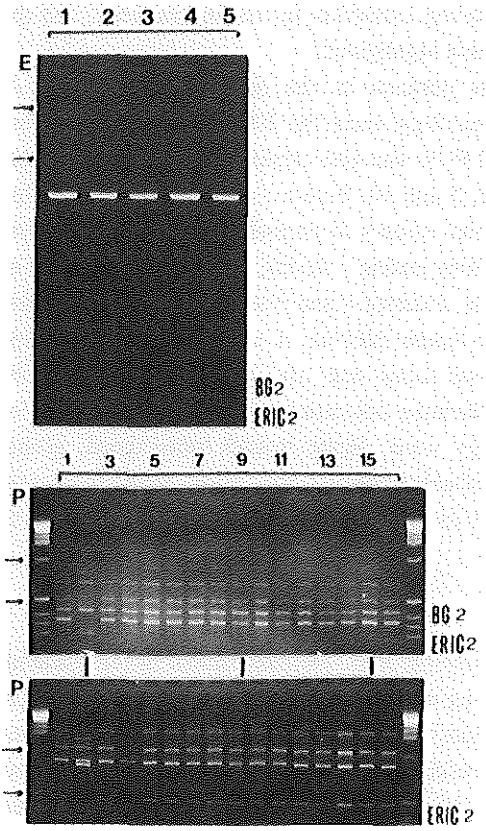


Figure 2 Examples of PCR fingerprints generated with *C. albicans* DNA samples from serial *C. albicans* isolates from BMT patients. Application of the BG2/-ERIC2 PCR to the five strains from patient E, isolated during a two month surveillance period and originating from several anatomical locations reveals the uniformity of the patterns obtained. In case of patient P (lower panels) the abberant isolate (lane 2) is obvious (see also Results). This is confirmed with both the BG2/ERIC2 PCR and the ERIC2 PCR. Numbering above the lanes indicates sample numbering in the order of appearance in Figure 1. Arrows on the left indicate molecular length markers. The upper arrow points at a 1000 bp fragment, the lower one at a 500 bp DNA molecule.

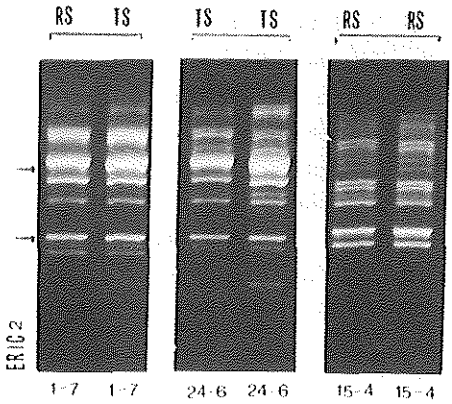


Figure 3 Examples of PCR fingerprints generated with *C. albicans* DNA samples from dual isolates from haemato-oncology patient 3, 4 and 5. The ERIC2 PCR was applied, TS and RS indicate throat and rectal swabs, respectively. The arrows on the right point at 1000 and 500 basepairs length markers.

in this patient. Figure 2 illustrates some of the results obtained with primers BG2/ERIC2 and ERIC2 alone. The pictures show the observed variability in banding patterns, and the aberrant isolates deriving from patient P are also highlighted.

Typing of *C. albicans* strains from patients in the oncology ward: For all patient isolates deriving from the haemato/oncology ward the ERIC2 assay was performed. For all patients except patient 3 the 799 PCR was also carried out. The 799/944 and BG2/ERIC2 were performed for patients 2, 4, 5 and 10. Figure 3 gives examples of some of the results obtained for the duplicate samples isolates from patients 3, 4 and 5. Again, these patients appear to be colonised by "long-lasting" strains. Patient 3 harbours an identical strain in the rectum and in the throat. Patients 4 and 5 still have the same strain in the throat and rectum monitored at a one-month interval. A similar result was found for patient 1. Patient 2, however, harboured rectal and oral strains which could be identified quite easily (results not shown), indicating colonisation by multiple strains. For the 35 individuals from the haemato/oncology ward, single isolates were typed, giving rise to at least 20 genotypically distinguishable strains. The types most frequently encountered in this group were not identical to any one type found in the group of *C. albicans* strains from the BMT patients.

DISCUSSION

The usefulness of PCR-mediated genotyping of *C. albicans* strains in the epidemiological monitoring of spread among and long-term colonisation of neutropenic patients was studied. It appeared from pilot experiments that in juvenile BMT patients hardly any variation in individual colonisation was encountered when single clones (colonies) from the primary clinical culture were analyzed. All clones displayed identical PCR finger-

prints. This implies that a maximum of 2% of genetic variants are present within a population. When these same patients are monitored for a prolonged period of time, again no new variants are encountered, irrespective of the elaborate treatment protocols the patients receive. The different anatomical locations monitored contained identical strains, when individual BMT patients are considered. No variation in colonisation was found and although we cannot determine which of the infections were nosocomially acquired, the estimation is that patient-to-patient infection rates are low. The fact that all BMT isolates could be differentiated by PCR fingerprinting is in favour of this assumption, which is in agreement with recent data on colonisation of children in a Leeds Hospital (20). However, another report concludes that significant patient-to-patient transmission of *Candida* strains may occur (25). Most probably, these differences are a result of the implementation of certain hospital hygiene measures. It is reassuring to notice that treatment with fungostatic compounds does not affect the nature of the colonising strains. The use of these compounds does not lead to selection of "new" strains or complete clearance followed by recolonisation by externally acquired *C. albicans* types. This is independent of the presence of neighbouring patients of the haemato-oncology ward (outside of the BMT unit), carrying other *C. albicans* strains, implying that either hospital control measures are adequate or reinfection or recolonisation by new strains is prevented by the presence of a strain that already occupies the ecological niches present in a given patient. An important conclusion is that, although trafficking between BMT units by medical personnel is frequent, transmission of *C. albicans* is rare or even absent in the Liverpool setting.

From a laboratory technical point of view, PCR fingerprinting is a simple and efficient means for discriminating fungal isolates. Given an operational PCR laboratory, more than hundred isolates can be typed by several PCR tests within a week. For typing of fungi, several DNA- or protein-based systems have been described (15) and adequate resolution of strains, enabling epidemiological evaluation, can be achieved. When typing methods are compared, advantages and disadvantages are encountered. Some of the epidemiological typing techniques is technically complex and requires sophisticated skills and equipment and have inadequate discriminatory power (for a recent review on technological aspects see reference 12). For typing of *C. albicans* it is generally advised to combine techniques for maximum reliability (11, 13). For this reason, we recently compared PCR typing, as described here, with some conventional techniques (24). It appeared that combination of PCR tests allowed discrimination of strains as well as RFLP analysis using multiple enzymes, pulsed field gel electrophoretic karyotyping and probing Southern blots with a repeat-specific DNA probe. Thus, the discriminatory power of PCR fingerprinting is as good as that of the more established techniques and this approach is generally less complex and more versatile.

In conclusion, it can be stated that PCR fingerprinting provides a simple and novel approach for monitoring the dynamics of nosocomial colonisation by a fungal species such

as *C. albicans*. The procedure described here can be extrapolated to other microbial pathogens involved in hospital infections. As such, PCR fingerprinting holds promise for future analyses of epidemics and incidental infections. The problems concerning automation, interlaboratory standardisation and data processing require resolution, but densitometers and sophisticated software will hopefully soon circumvent these problems.

REFERENCES

1. **Anaissie E** (1992) Opportunistic mycoses in the immunocompromised host: experience at a cancer center. Clin. Infect. Dis. **14**: 543-553.
2. **Arnaro P.M., M. Sadigh, C. Costas, D. Weil and R. Chudy** (1991) Endemic and epidemic aspergillosis associated with in-hospital replication of *Aspergillus* organisms. J. Infect. Dis. **164**: 998-1002.
3. **Bingen E., E. Denamur, N. Lambert et al** (1992) Mother-to-infant vertical transmision and cross-colonisation of *Streptococcus pyogenes* confirmed by DNA RFLP analyes. J. Infect. Dis. **165**: 147-150.
4. **Bloom B.R. and C.J.L. Murray** (1992) Tuberculosis: commentary on a re-emergent killer. Science **257**: 1055-1064.
5. **Blumberg H.M., D. Rimland, J.A. Kielbauch, P.M. Terry and I.K. Wachsmuth** (1992) Epidemiologic typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphisms of rRNA genes: Elucidation of the clonal nature of bacteriophage-nontypable, ciprofloxacin-resistant, methicillin-susceptible *S. aureus* isolates. J. Clin. Microbiol. **30**: 362-369.
6. **Boom R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim van Dillen and J. van der Noordaa** (1990) Rapid and simple method for the purification of nucleic acids. J. Clin. Microbiol. **28**: 495-503.
7. **De Bruin F.J** (1992) Use of repetitive sequences and the PCR to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Envir. Microbiol. **58**: 2180-2187.
8. **Fraser V.J., M. Jones, J. Dunkel, S. Storfer, G. Medoff and W.C. Dunagan** (1992) Candidemia in a tertiary care hospital: Epidemiology, risk factors and predictors of mortality. Clin. Infect. Dis. **15**: 414-421.
9. **Jayaro B.M., B.J. Bassam, G. Caetano-Anoles, P.M. Gresshoff and S.P. Oliver** (1992) Subtyping *Streptococcus uberis* by DNA amplification fingerprinting. J. Clin. Microbiol. **30**: 1347-1350.
10. **Jeffreys A.J., A. Maceod, K. Tamaki, D.L. Neil and D.G. Monckton** (1991) Minisatellite repeat coding as a digital approach to DNA typing. Nature **354**: 204-209.
11. **Magee, P.T., L. Bowdin and J. Staudinger** (1992) Comparison of molecular typing methods for *C. albicans*. J. Clin. Microbiol. **30**: 2674-2679.
12. **Maslow, J.N., M.E. Mulligan and R.D. Arbeit** (1993) Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. Clin. Infect. Dis. **17**: 153-164.
13. **Odds, F.C., D.L. Brawner, J. Staudinger, P.T. Magee and D.R. Soll** (1992) Typing of *Candida albicans* strains. J. Med. Vet. Mycol. **30**: 87-94.

14. **Patton C.M., I.K. Wachsmuth, G.M. Evins et al** (1991) Evaluation of 10 methods to distinguish epidemic associated *Campylobacter* strains. J. Clin. Microbiol. **29**: 680-688.
15. **Pfaller M.A** (1992) Epidemiological typing methods for mycoses. Clin. Infect. Dis. **14**: 59-510.
16. **Piffaretti J., H. Kressebuch, M. Aeschbacher et al** (1989) Genetic characterisation of clones in the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. USA **86**: 3818-822.
17. **Perfect J.R., W.W. Pickard, D.C. Hunt, B. Palmer and W.A. Schnell** (1991) The use of Amphotericin B in nosocomial fungal infection. Rev. Infect. Dis. **13**: 474-479.
18. **Quin P.A., J.E. Gillan, T. Markestad et al** (1985) Intrauterine infection with *Ureaplasma urealyticum* as a cause of fatal neonatal pneumonia. Ped. Infect. Dis. **4**: 538-543.
19. **Schmid J., F.C. Odds, M.J. Wiselka, K.G. Nicholson and D.R. Soll** (1992) Genetic similarity and maintenance of *Candida albicans* strains from a group of AIDS patients, demonstrated by DNA fingerprinting. J. Clin. Microbiol. **30**: 935-941.
20. **Sharp A.M., F.C. Odds and E.G.V. Evans** (1992) *Candida* strains from neonates in a special care baby unit. Arch. Dis. Childh. **67**: 48-52.
21. **Still T.C., J.J. Lipuma and T.D. Edlind** (1988) A broad spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. J. Infect. Dis. **157**: 280-286.
22. **Talbot G.H., A. Huang and M. Provencher** (1991) Invasive aspergillosis in patients with acute leukemia. J. Infect. Dis. **13**: 219-232.
23. **Van Belkum A., M. Struelens and W. Quint** (1992) Epidemiologic typing of *Legionella pneumophila* by PCR mediated genome fingerprinting with arbitrary and repeat sequence primers. J. Clin. Microbiol. **31**: 2198-2200.
24. **Van Belkum, A., W. Melchers, B.E. de Pauw, S. Scherer, W. Quint and J.F.G.M. Meis** (1994) Genotypic characterisation of sequential *Candida albicans* isolates from fluconazole-treated neutropenic patients. J. Infect. Dis. **169**: 1062-1070.
25. **Vazquez, J.A., V. Sanchez, C. Dmuchowski, L.M. Dembry, J.D. Sobel and M.J. Zervos** (1993) Nosocomial acquisition of *Candida albicans*: an epidemiological study. J. Infect. Dis. **168**: 195-201.
26. **Versalovic J., T. Koeuth and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19**: 6823-6831.
27. **Vogt P** (1990) Potential genetic function of tandem repeated sequence blocks in the human genome are based on a highly conserved chromatin folding code. Hum. Genet. **4**: 301-336.
28. **Welsh J. and M. McClelland** (1991) Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. Nucleic Acids Res. **19**: 5275-5279.
29. **Williams J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey** (1991) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18**: 6531-6535.

Adapted from: J. Clin. Microbiol. 1993;31:2502-2505

CHAPTER VIII

TYPING OF *ASPERGILLUS* SPECIES AND *ASPERGILLUS FUMIGATUS* ISOLATES BY INTERREPEAT POLYMERASE CHAIN REACTION

Alex van Belkum, Wim G.V. Quint[1], Ben E. de Pauw[2], Willem J.G. Melchers[3] and Jacques F. Meis[3]

[1]Diagnostic Center SSDZ, P.O. Box 5010, 2600 GA Delft, The Netherlands. [2]Department of Hematology and [3]Department of Medical Microbiology, University Hospital, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

SUMMARY

PCR amplification of repetitive DNA motifs allows discrimination of *Aspergillus* species. In a preliminary survey, the DNA fingerprints appear to be identical when isolates of *Aspergillus fumigatus* are compared. When a primer deduced from a prokaryotic repeat motif is used, *Aspergillus fumigatus* isolates originating from different patients or different anatomical locations can be typed individually.

INTRODUCTION

Invasive infections caused by fungi of the genus *Aspergillus* are a problem in immunocompromised patients (6,9). In particular, *Aspergillus fumigatus* is a frequently encountered pathogen in neutropenic patients (7). Major sites of infection are the lungs, from which systemic infections originate (14). Invasive aspergillosis is difficult to diagnose during life, and successful treatment depends on the stage of fungal proliferation and degree of neutropenia (5). It is for these reasons that an improvement of the present, insufficient means for *Aspergillus* detection is required (13). This also concerns the identification and typing of isolates. Knowledge of the genetic characteristics of a fungal isolate allows epidemiological analysis of spreading and detection of the putative origin of pathogenic strains (3). DNA amplification methods enable this kind of genetic typing. Amplification of random polymorphic DNA elements (RAPD typing) allows distinction between isolates of *A. fumigatus* (1). The present study describes the applicability of polymerase chain reaction (PCR) assays aiming at repetitive and intrinsically variable DNA sequences for identification of *Aspergillus* species and *A. fumigatus* isolates.

MATERIALS AND METHODS

Aspergillus strains and several clinical isolates were used for DNA isolation and typing. Reference strains for *A. fumigatus, A. terreus, A. niger, A. nidulans* and *A. flavus* were obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Clinical isolates were obtained from samples from neutropenic patients. All species and isolates are described in Table 1. DNA was prepared from fungal cultures in liquid medi-

Table 1 Summary of data on fungal strains and infected patients

Strain	Species	Code	Patient	Clinical material
1	*Aspergillus terreus*	CBS106.25	NA	NA
2	*Aspergillus niger*	CBS102.12	NA	NA
3	*Aspergillus nidulans*	CBS100.20	NA	NA
4	*Aspergillus fumigatus*	CBS113.26	NA	NA
5	*Aspergillus flavus*	CBS108.30	NA	NA
286	*Aspergillus terreus*	423984	A	wound smear
299	*Aspergillus terreus*	427793	A	wound smear
308	*Aspergillus versicolor*	427611	B	faeces
312	*Aspergillus fumigatus*	429324	B	faeces
317	*Aspergillus fumigatus*	430005	B	BAL
408	*Aspergillus fumigatus*	451216	B	lung tissue
409	*Pseudoallescheria boydii*	448697	C	lung biopsy
410	*Rhizopus microsporus*	452772	D	stomach tissue
441	*Fusarium spec.*	464450	E	BAL
466	*Aspergillus fumigatus*	470519	F	lung biopsy
487	*Aspergillus fumigatus*	479611	G	sputum
489	*Aspergillus fumigatus*	480680	H	sputum
496	*Aspergillus fumigatus*	484482	I	lung biopsy
509	*Aspergillus flavus*	486750	J	BAL
512	*Aspergillus flavus*	488196	J	pericard section

Note: CBS codes are identification numbers as used within the Centraalbureau voor Schimmelcultures, the other codes are serial numbers from the Medical Microbiology Department, University hospital, Nijmegen, the Netherlands. Patients are encoded A through J, BAL represents bronchoalveolar lavages. Isolate 441 concerns a *Fusarium* isolate, which has not been identified down to the species level. Strain numbers correspond to lane numbering in Figures 1 to 3. Patient codes A to J are also shown in Figures 2 and 3; NA: not applicable.

um (brain heart infusion broth) by a combination of mechanical, enzymatic and chemical procedures. Cultures were concentrated by centrifugation or filtration and the biomaterial was resuspended in 100 μl of 1M sorbitol, 25 mM K_2HPO_4 pH 7.8, 10 mM $MgCl_2$, 2 mM dithiothreitol. The material was homogenised by using a small pestle, exactly fitting the Eppendorf tubes. After the addition of 25 μl of a solution of lysing enzymes from *Trichoderma harzianum* (10 mg/ml, Sigma) in phosphate-buffered saline pH 7 (PBS), incubation was carried out at 30°C for 60 min. Subsequently, 15 μl of 10% Triton X-100 in water was included, and the flocculent fungal remains were homogenised once more

with a pestle. From the homogenate, DNA was isolated through guanidinium isothiocyanate lysis and DNA binding to Celite, according to previously described procedures (2). Reaction mixtures for PCR contained 50 ng of DNA and consisted of 10 mM Tris.HCl pH 9, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM deoxyribonucleotide triphosphates, and 0.5 units of Taq DNA polymerase (Super Taq; Sphaero Q, Leiden, The Netherlands). PCR was performed by using one of two 40- cycle programs consisting of denaturation (1 min 94°C), annealing (1 min 25°C or 2 min 55°C, for program I or II, respectively) and DNA chain extension (2 min or 3 min 74°C, program I or II respectively) in a Biomed thermocycler (model 60). Primers were present at a concentration of 0.5 μM. A survey of primers used is shown in Table 2.

Table 2 Survey of PCR primers applied in fungal DNA typing

primer ref. number	nature	DNA sequence	
738	dimeric simple sequence motif	ACACACACACACACACAC	(7)
785	trimeric simple sequence motif	CACCACCACCACCACCACCAC	(7)
799	yeast telomeric consensus sequence	TGGGTGTGTGGGTGTGTGGGTGT	(7,9)
800	ribosomal spacer repeat	GACAGACAGACAGACAGACA	(7,9)
944	trimeric simple sequence motif	GGAGGAGGAGGAGGAGGAGGA	(9)
1014	ERIC1	ATGTAAGCTCCTGGGGATTCAC	(10)

Note: Primers 738, 785, 799, 800 and 944 were used in combination with PCR program II, whereas primer 1014 was always used in combination with PCR program I.

RESULTS

The results of PCR amplification of loci bordered by repetitive motifs present in the genome of different *Aspergillus* species are displayed in Figure 1. DNA from the five species examined can be discriminated, independently of the primer used. This interrepeat PCR (IR PCR) apparently leads to the generation of species-specific PCR fingerprints. DNA fragments of between 400 and 2000 basepairs in length are synthesised. Analysis of a group of 15 clinical fungal isolates with another simple sequence motif primer (944) confirmed the aforementioned observations (Figure 2). Also, other genera and species such as *Fusarium* spp, *Aspergillus versicolor*, *Pseudoallescheria boydii* and *Rhizopus microsporus*, can be identified on the basis of a specific banding pattern. Interestingly, all *A. fumigatus* DNA preparations derived from five different patients gave rise to an identical DNA banding pattern. Assuming that this also applies to larger numbers of isolates, this finding implies that on the basis of the exact nature of this fingerprint, *A.*

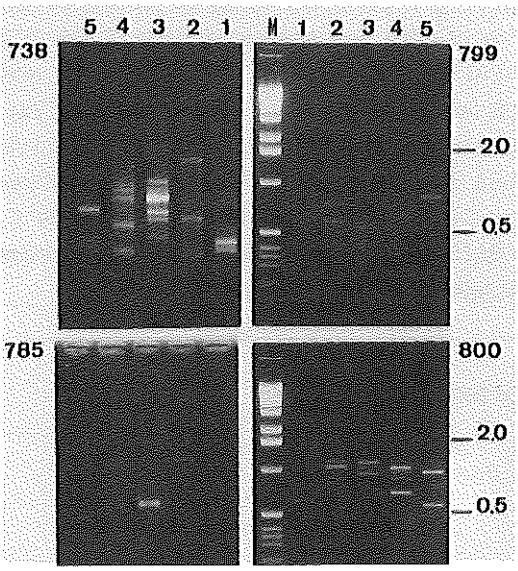
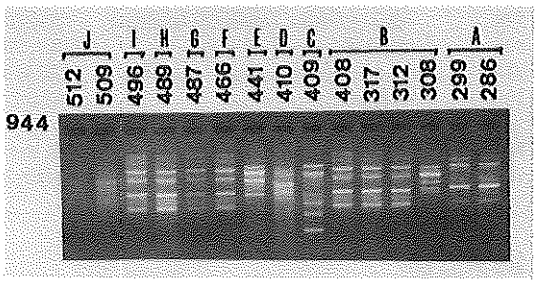


Figure 1 Interrepeat PCR of genomic DNA from five clinically relevant *Aspergillus* species. Lane numbering (1 to 5) corresponds with *A. terreus, A. niger, A. nidulans, A. fumigatus* and *A. flavus* DNA, respectively. The primer numbers are indicated beside each panel, primer description is given in Table 2. On the right, the length of two of the molecular weight marker molecules (1 kb ladder, GIBCO BRL)-(lane M) is indicated in kilobasepairs.

Figure 2 Interrepeat PCR on genomic DNA from fifteen clinical isolates of seven fungal species. The primer used (944) has a nucleotide sequence resembling a trimeric simple sequence motif. Isolate codes corresponding to those listed in Table 1 and patient codes (A through J) are indicated above the lanes.



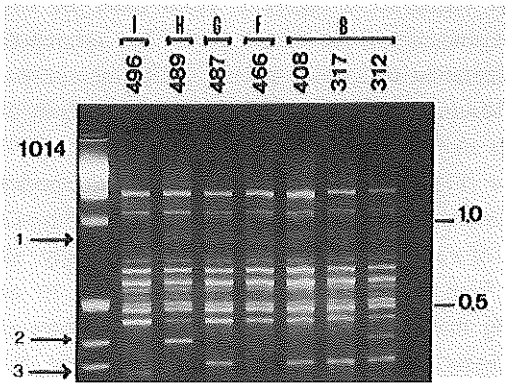


Figure 3. Interrepeat PCR on genomic DNA from clinical *A. fumigatus* isolates. Strain numbering and patient codes (letters B to I) correspond to those given in Table 1, primer 1014 is characterised in Table 2. On the left, the major PCR fragment length polymorphisms are indicated (arrow 1 to 3). On the right the molecular weight marker is indicated.

fumigatus could be identified to the species level. Also, isolates of *A. terreus* (patient A) and *A. flavus* (weak banding pattern, patient J) display constant PCR fingerprints. When the eukaryotic repeat motif primers are displaced by prokaryotic repeat-like primers, another picture arises (Figure 3). It appears that alteration of the PCR conditions and primers gives rise to isolate-specific PCR fingerprints. The arrows (1 to 3, Figure 3) indicate polymorphic sites, on the basis of which *A. fumigatus* isolates can be discriminated. For isolates 487 and 317, the difference is minor: a small difference in migrational distance can be observed only at the position of arrow 1. Isolates 317 and 408 are apparently identical, probably because of the fact that these strains originated from the same anatomical location within a single patient. Isolate 317 was cultured from bronchoalveolar lavage fluid, whereas isolate 408 originated from postmortem lung tissue from a patient with a histologically confirmed diagnosis of invasive aspergillosis. It is interesting that the two fungal isolates derived from this patients' faeces (isolates 308 and 312) either belong to a different species (308: *A. versicolor*) or display an aberrant PCR fingerprint (isolate 312: additional 400 basepair DNA fragment, Figure 3). The reproducibility of the PCR fingerprinting procedure is good. It has to be mentioned, however, that upon using a newly synthesised batch of PCR primers (especially in case of the prokaryotic repeat primers) the banding patterns may vary slightly. These minor changes, however, do not affect the discriminatory power of the procedure. This problem can be overcome by the usage of high-performance liquid chromatography (HPLC) purified oligonucleotides (unpublished observations).

DISCUSSION

A large proportion of the genome of eukaryotes consists of repetitive DNA. Many of these motifs display a strong tendency towards variation and can be used for the identification of individuals on the basis of their sometimes extreme degree of genetic variability (12). This knowledge led to the use of these repeat motifs for PCR-mediated discrimination of DNA extracted from lower eukaryotes (10). The present communication describes the use of these PCR primers for distinguishing fungi, with emphasis on molds from the genus *Aspergillus*. It appears that interrepeat PCR discriminates among fungal species and, as such, provides a new method for species identification. It has been noticed that various yeast species can also be very adequately identified using similar procedures (8). It has to be emphasised that a rather limited selection of strains was studied. In order to make definite statements concerning the potential of this type of PCR for the determination of the nature of a species, more-extensive studies involving strains from more-diverse geographical origins should be performed. Also, this procedure does not detect DNA polymorphisms between isolates of a single species, which is a prerequisite for epidemiological application of PCR fingerprinting. In order to overcome this shortcoming, an attempt was initially made to increase the variability of the fingerprinting strategy by combining several of the primers (Table 2) in a single amplification reaction.

This led to an increase in the number of DNA fragments synthesised, but no differences between non-related isolates of one species could be distinguished (results not shown). Therefore, prokaryotic repeat motif primers were applied to achieve this goal. It has been demonstrated that these PCR primers can be successfully applied in the genotypic characterisation of even very closely related strains of a multitude of bacterial species (11). In the case of *A. fumigatus* (Figure 3) the same approach appeared to be successful. Six of seven clinical isolates could be discriminated, the two identical isolates originated from the same anatomical location in a single patient. For this patient, it was established that the faecal isolates cultured one week before obtaining the bronchoalveolar lavage were genetically different from the invasive strain. This simple example illustrates the clinical and epidemiological value of genetic typing of *A. fumigatus* (see also references 1 and 3). This finding, in combination with the observation that the prokaryotic repeat primer does not seem to cross-react with human DNA, leads to the cautious assumption that the typing procedure may eventually be directly suited for the analysis of clinical material itself. It has recently been demonstrated that the DNA fragments generated by PCR fingerprinting may represent species-specific DNA probes (4). These developments may speed up the establishment of PCR fingerprinting-mediated detection-identification systems.

REFERENCES

1. **Aufauvre-Brown A., J. Cohen, and D.W. Holden** (1992) Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. **30**, 2991-2993.
2. **Boom R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim and J. Van der Noordaa** (1990) Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28**, 495-503.
3. **Denning D.W., K.V. Clemons, L.H. Hanson, and D.A. Stevens** (1990) Restriction endonuclease analysis of total cellular DNA of *Aspergillus fumigatus* isolates of geographically and epidemiologically diverse origin. J. Infect. Dis. **162**, 1151-1158.
4. **Giesendorf B.A.J., A. van Belkum, A. Koeken, H. Stegeman, M.H.C. Henkens, J. van der Plas, H.G.M. Niesters, W.G.V. Quint** (1993) Development of species-specific DNA probes for *Campylobacter jejuni, Camplobacter coli* and *Campylobacter lari* by Polymerase Chain Reaction fingerprinting. J. Clin. Microbiol. **31**, 1541-1546.
5. **Graybill J.R** (1992) Future directions of antifungal chemotherapy. Clin. Infect. Dis. **14**, suppl 1, S170-181.
6. **Kusne S., J. Torre, R. Manez, W. Irish, M. Martin, J. Fung, R.C. Simmons and T.E. Starzl** (1992) Factors associated with invasive lung aspergillosis and the significance of positive *Aspergillus* culture after liver transplantation. J. Infect. Dis. **166**, 1379-1383.
7. **Meis, J.F., J.P. Donneley, S.A. Hoogkamp-Korstanje and B.E. de Pauw** (1993) *Aspergillus fumigatus* pneumonia in neutropenic patients during therapy

with Fluconazole for infection due to *Candida* species. Clin. Infect. Dis. **16**, 1812-1816.

8. **Niesters H., W. Goessens, J. Meis and W.G.V. Quint** (1993) Rapid PCR mediated assays for identification of pathogenic fungi. J. Clin. Microbiol. **31**, 903-908.
9. **Talbot GH, A Huang, and M Provencher** (1991) Invasive *Aspergillus* rhinosinusitis in patients with acute leukemia. Rev. Infect. Dis. **13**, 219-232.
10. **Van Belkum A., J. De Jonckheere J., and W.G.V. Quint** (1992) Genotyping *Naegleria* spp. and *Naegleria fowleri* isolates by interrepeat PCR. J. Clin. Microbiol. **30**, 2595-2598.
11. **Versalovic J., T. Koeuth, and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res **19**, 6823-6831.
12. **Warburton P.E., and H.F. Willard** (1992) PCR amplification of tandemly repeated DNA: Analysis of intra- and interchromosomal sequence variation and homologous unequal crossing over in human alpha satellite DNA. Nucleic Acids Res **20**, 6033-6042.
13. **Warnock D.W., A.B.M. Foot, E.M. Johnson, S.B. Mitchell, J.M. Cornish and A. Oakhill** (1991) *Aspergillus* antigen latex test for diagnosis of invasive aspergillosis. Lancet **338**, 1023-1024.
14. **Young R.C., J.E. Bennett, C.L. Vogel, P.P. Carbone and V.T. Devita** (1970) Aspergillosis: the spectrum of the disease in 98 patients. Medicine **49**: 147-173.

Adapted from: J. Microbiol. Meth. 1994;20:235-247

CHAPTER IX

PCR FINGERPRINTING FOR EPIDEMIOLOGICAL STUDIES OF *STAPHYLOCOCCUS AUREUS*

Alex van Belkum, René Bax[1], Piet J.C. van der Straaten[2], Wim G.V. Quint[1] and Etel Veringa[3]

[1]Diagnostic Centre SSDZ, Dept. of Molecular Biology, P.O. Box 5010, 2600 GA Delft, the Netherlands. [2]Reinier de Graaf Gasthuis, Dept. of Pediatric Diseases, P.O. Box 5012, 2600 GA Delft, the Netherlands. [3]Diaconessenhuis, Department of Medical Microbiology, Houtlaan 55, 2334 CK Leiden, the Netherlands.

SUMMARY

***Staphylococcus aureus* isolates (n=126), collected during two different periods from patients hospitalised in paediatric wards, were analyzed using polymerase chain reaction (PCR) mediated genotyping. These isolates were compared with 29 isolates from individuals attending the out patient clinic of the same hospital and 13 isolates from paediatric hospital personnel. Within a group of 99 isolates gathered from 48 individuals during surveillance period I, 22 distinct genotypes were identified by application of two PCR assays. Among the 58 isolates collected in surveillance period II from paediatric and out-clinic patients, 25 genotypes were detected by a single PCR assay only. Based on these results it was demonstrated that patients can be colonised with multiple strains that may persist in a certain anatomical location for prolonged periods of time. It is shown that persistence of a *S. aureus* strain in a paediatric ward can be deduced from the PCR genotyping studies. As such PCR can be used for longitudinal monitoring of bacterial infections in hospital departments, analysis of patient-to-patient and personnel-to-patient transmission and for detection of genetic variation in general in *S. aureus*. Also, isolate-specific DNA probes can be generated for *S. aureus* by PCR genotyping. The probes can be used for the recognition of re-emerging *S. aureus* epidemics.**

INTRODUCTION

Despite preventive measures, hospital infections remain a difficult problem, which may result in morbidity, prolonged hospitalisation and increased costs. Rapid and discriminative tests for identification and comparison of different isolates are essential for localisation of sources and prevention of spread of nosocomial infections. The presently available molecular tests are complex, laborious and time-consuming and beyond the means of a standard laboratory. DNA typing assays have been evaluated but for these tests relatively large amounts of high molecular weight DNA are needed, apart from other practical drawbacks. Polymerase chain reaction (PCR) mediated genotyping assays (4) provide an attractive alternative system. It is as discriminative as other molecular typing assays (1, 7, 10) and the total number of PCR tests that can be performed on a single

TABLE 1A Survey of *S. aureus* infections monitored between July and November 1991, Pediatric Dept, RdGG Delft

patient nr	sample number	material	genotype species 1 2 3 4 5	patient	sample number	material	genotype species 1 2 3 4 5
Surveillance period I							
Pediatric patients				**Pediatric patients**			
1	22197	ear	A A - - -	32	15634	navel	A A - - -
2	19599	nose	F I - - -	32	15633	nose	B E - - -
2	19597	throat	F I - - -	32	15632	throat	B D - - -
2	19596	navel	B H - - -	32	15631	ear	B D - - -
3	18250	ear	A A - - -	33	15371	eye*	A A - - -
5	18145	eye*	A A - - -	34	24288	ear	A A - - -
5	18144	navel[@]	A A - - -	34	24287	nose	A A - - -
6	17326	throat	D F - - -	34	24286	navel	A A - - -
6	17325	nose	E G - - -	35	15414	nose	A A - - -
6	17324	ear	E G - - -	35	16120	nose	A A - - -
6	17323	navel	E G - - -	35	16129	nose	A A - - -
7	23925	nose	A A - - -	38	25946	nose	B O - - -
7	23922	throat	I K - - -	38	25945	throat	B O - - -
7	23921	navel	I K - - -	39	17601	nose	A A - - -
8	16646	throat	B B - - -	39	17600	throat	A A - - -
8	16645	nose	B B - - -	39	17599	navel	A A - - -
8	16643	navel	B B - - -	39	17598	ear	A A - - -
9	19892	navel	B D - - -	40	21703	nose	A A - - -
10	21940	ear	B B - - -	41	21966	nose	F A - - -
10	21939	navel	B B - - -	41	21964	navel	F A - - -
10	21938	nose	B B - - -	42	24043	ear	A A - - -
10	11937	throat	B B - - -	42	24042	nose	A A - - -
10	22036	navel[@]	B B - - -	42	24041	throat	A A - - -
13	14947	navel	C C - - -	42	24040	navel	B K - - -
13	14946	ear	C C - - -	43	15451	nose	B B - - -
13	14945	nose	C C - - -	43	16121	nose	B B - - -
13	14944	throat	B B - - -	43	16127	nose	B B - - -
14	18723	navel	B B - - -	44	16730	nose	D F - - -
14	18722	nose	B B - - -	44	16729	throat	D F - - -
14	18721	ear	B B - - -	45	25439	throat	C A - - -
15	18477	eye*	A A - - -	46	21525	navel	C C - - -
15	19010	eye*	A A - - -	46	21523	nose	G C - - -
16	18287	throat	A A - - -	48	24895	nose	B M - - -
16	52074	blood[‡]	B P - - -	48	24893	ear	B M - - -
16	18498	navel[$]	B D - - -				
17	23425	navel	F K - - -	**Children's department**			
18	17440	nose	B B - - -	4	17950	nose	A A - - -
19	17044	ear	B D - - -	11	23258	nose	F A - - -
19	17043	nose	B D - - -	12	15801	nose	A A - - -
20	18291	navel	C C - - -	24	14665	navel	A A - - -
21	19612	eye*	B D - - -	25	24950	throat	C N - - -
22	23277	nose	H L - - -	27	22207	nose	F K - - -
22	23275	throat	H L - - -	28	18410	throat	B B - - -
23	21133	nose	B B - - -	30	19021	nose	A A - - -
23	21132	throat	B B - - -	36	17369	eye	B D - - -
26	14953	nose	A A - - -	36	17366	nose	B D - - -
26	14952	ear	B B - - -	36	17437	pus	B D - - -
29	25343	navel	B K - - -	37	18823	nose	F B - - -
29	25336	nose	A A - - -	47	21489	nose	G J - - -
29	25773	throat	A A - - -				
29	25772	nose	A A - - -				
31	15646	navel	B D - - -				

Note: The sample number indicates laboratory registration. Numbers 1 to 48 were patients admitted to the neonatology ward, number 4 to 47 (bottom part of right column) denominate 13 patients in the children's department. The genotypic code combines the codes as deduced from fingerprinting assays (*infected eye; [@]infected navel; [‡]exchange blood transfusion; [$]catheter). Genotypes 1 to 5 are derived from PCRs involving primers 1246, 935/1236, 935/1122, 1246 and 1220, respectively.

TABLE 1B Survey of *S. aureus* infections monitored between October and December 1992, Pediatric Dept. RdGG Delft

patient nr		sample number	material	genotype species 1 2 3 4 5	patient nr	sample number	material	genotype species 1 2 3 4 5
Pediatric patients					**Out-clinic patients**			
1	A	217121	nose	- - A A A	21	I	217791	drain	- - G H H
2	A	217111	throat	- - A A A	22	II	21871	ear	- - B B I
3	A	21749	tube	- - A A A	23	III	21914	knee	- - C H J
4	B	215322	stomach	- - B B B	24	IV	21975	leg	- - B I K
5	C	214651	groin	- - A A A	25	V	220641	perianal	- - B I K
6	D	21203	nose	- - C D D	26	VI	218672	urine	- - C D L
7	D	21211	blisters	- - A A A	20	VII	23073	nose	- - - - N
8	D	212081	navel	- - A A A	29	VIII	23075	mouth	- - - - O
9	D	22097	ear	- - C D D	30	IX	24015	wounded leg	- - - - A
10	D	22096	nipple*	- - C D D	31	IX	24014	wounded leg	- - - - A
11	D	22086	pus	- - A A A	32	X	24006	elbow	- - - - P
12	E	215271	nose	- - B E E	35	XI	22309	wounded leg	- - - - N
13	E	215261	ear	- - E F F	36	XII	23236	skin	- - - - B
14	E	215242	sputum	- - F G G	37	XIII	23243	nose	- - - - Q
15	F	21563	sputum	- - B B B	38	XIV	23738	pink	- - - - R
16	G	21968	nose	- - B B B	39	XV	23733	ear	- - - - R
17	G	21967	navel	- - B B B	40	XVI	23919	fistel	- - - - S
18	G	21995	penis	- - Ni B B	41	XVII	23878	anus	- - - - T
19	G	21966	throat	- - B B B	42	XVIII	23649	foot	- - - - U
20	G	21995	penis	- - A B B	43	XIX	23737	foot	- - - - V
33	H	24047	navel	- - - - Y	44	XVIII	23649	foot	- - - - N
34	H	24045	pus	- - - - Y	45	XX	23233	arm	- - - - N
52	H	24044	nose	- - - - Y	46	XXI	23235	ankle	- - - - N
54	I	23638	navel	- - - - R	47	XXII	23531	hand	- - - - C
54	J	22974	nose	- - - - O	48	XXIII	23163	leg	- - - - N
55	J	22976	navel	- - - - C	49	XXIV	23539	groin	- - - - W
56	K	23025	unknown	- - - - R	50	XXV	23437	wound	- - - - N
57	L	22590	throat	- - - - C	31	XXVI	23312	throat	- - - - X
					58	XXVII	23202	ear	- - - - B

NB. The sample number (5/6 digits) indicates laboratory registration. Patients A through L were admitted to the neonatology ward; patients I through XXVII were seen in the out-clinic. The genotypic code contains the codes as deduced from individual fingerprinting assays. The first number corresponds to the numbering in Figure 2.

DNA preparation is nearly unlimited. PCR fingerprinting, which discriminates even closely related bacterial strains, appears to be very promising (1, 3, 7, 10, 11, 12). In the present paper we demonstrate that PCR mediated DNA typing of *Staphylococcus aureus* causing nosocomial infection is useful in epidemiological analyses. Moreover, the same methodology can be used for the isolation of *S. aureus* strain-specific DNA probes, the availability of which may facilitate the recognition of re-occurring epidemics.

MATERIALS AND METHODS

Patient populations and bacterial strains: In July 1991 several patients in the paediatric wards of Reinier de Graaf Gasthuis (Delft, The Netherlands) suffered from conjunctivitis caused by *S. aureus* infection. To establish a possible common source a general bacteriological surveillance was implemented (surveillance period I). In October 1992 a number of patients in the same wards developed skin blistering, again caused by *S. aureus*. Again a surveillance period (surveillance period II) started. During the first surveillance period of five months 99 isolates of *S. aureus* were collected from 48 patients from the paediatric neonatal ward and the daycare ward for older children (patients 1 to 48 and 4 to 47, respectively; Table 1A). During the same period 13 additional *S. aureus* isolates were obtained from 10 hospital employees. During the second surveillance period, 27 *S. aureus* strains were isolated from 12 neonates admitted to the neonatal ward within a period of two months (Table 1B, patients A through L). From several patients follow-up isolates were obtained during the second surveillance period. Also, twenty-nine *S. aureus* strains were isolated from 27 randomly selected individuals attending the out patient clinic (Table 1B, patients I through XVII).

In total 168 bacterial strains were collected. All isolates were identified with standard microbiological techniques such as catalase and coagulase reactions. Phenotypical methicillin sensitivity was demonstrated for all isolates by determination of the minimal inhibitory concentration, which appeared to be $\leq$ 1 mg/l. Prior to DNA extraction the isolates were incubated in Brain Heart Infusion (BHI) broth in a rotary shaker for at least 36 h at 37°C.

DNA isolation: A 500 μl sample of the *S. aureus* cultures in BHI medium was incubated in the presence of 25 μg proteinase K (Boehringer-Mannheim) and 0.5% sodium dodecyl sulphate (SDS) for 1 h at 37°C. The subsequent isolation and purification of DNA was performed using guanidinium-isothiocyanate and Celite according to established procedures (2). The resulting DNA solution was subjected to gel electrophoresis in 1% agarose gels containing 40 mM Tris.Acetate pH 7.8, 2 mM EDTA and 0.1 μg/ml ethidiumbromide. The DNA concentration was estimated by comparison with staining intensity of samples containing a known amount of bacteriophage lambda DNA (25-100 ng).

PCR primers and primer synthesis: Oligodeoxyribonucleotides were either synthesised on an Applied Biosystems DNA synthesizer model 381A and used without

further purification or the primers were purchased from Isogen (Amsterdam, The Netherlands). The concentration of the oligonucleotide solutions was determined by UV absorption measurements on a Uvicam spectrophotometer model PU 8720. Primer codes are *mec*A2 (935), BG2 (1122 or 1236), ERIC1 (1246) and ERIC2 (1220), representing oligos with the following base sequences respectively: 5'-AGTTCTGCAGTACCGGATTTGC-3', 5'-TACATTCGAGGACCCCTAAGTG-3', 5'-ATGTAAGCTCCTGGGGATTCAC-3' and 5'-AAGTAAGTGACTGGGGTGAGCG-5'. Figures between brackets denominate DNA synthesis batch numbers. Primers have been described before (1, 11).

PCR amplification of bacterial DNA: The reaction mixtures (100 μl) consisted of 10 mM Tris.HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100. Deoxyribonucleotide triphosphates were used in a 0.2 mM concentration. Per reaction 50 pmol of primer(s) and 0.5 unit Taq DNA polymerase (Super Taq, Sphaero-Q, Leiden, The Netherlands) was added. Template DNA (5 ng) were added in a 1 μl volume. PCR was performed using a 40-cycle programme consisting of consecutive denaturation (1 min 94°C), annealing (1 min 25°C) and DNA chain extension (2 min 74°C) in a Biomed thermocycler model 60. For analysis of reproducibility, for some of the strains (n=10), multiple primary colonies (n=20-50) were cultured and DNA extracted from these "clones" was subjected to PCR amplification. Serial dilutions of the DNA preparations were also studied, as were duplicate DNA preparations from independently grown but otherwise identical bacterial isolates. For all strains, between 1 and 3 different PCR tests were performed (Table 1A and B).

Purification and labelling of DNA probes: DNA fragments to be used as probes were cut from agarose gels and purified using Geneclean kits. Radiolabeling of these probes was performed using [α-^{32}P] dATP (Amersham, UK) and a random primed labelling procedure (5).

Southern blotting and hybridisation: PCR amplified and size separated DNA was transferred from agarose gels onto Hybond N^+ membranes (Amersham, UK) by vacuum blotting in the presence of 0.4 M NaOH. Hybridisation was performed using previously described procedures (8).

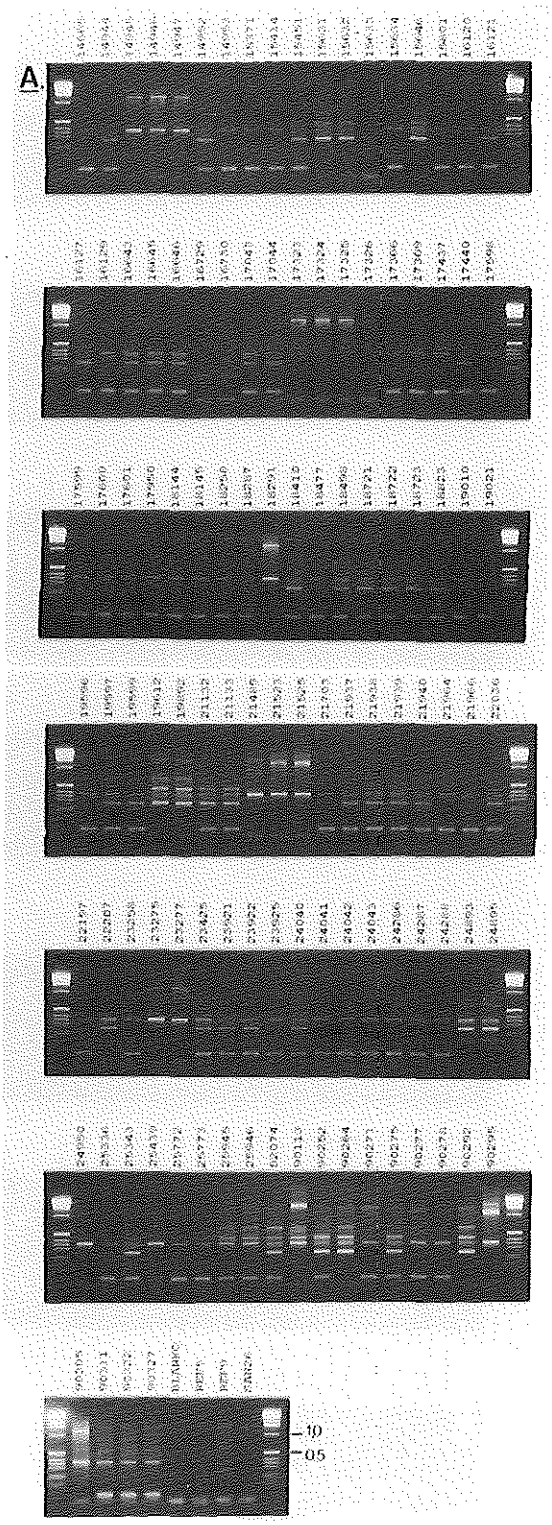
RESULTS

Reliability of PCR fingerprinting: When DNA banding patterns were compared for clones from single isolates, not a single difference was detected. Clear differences were seen when strain-specific banding patterns were analyzed. Identity included the minor bands seen in some of the PCR fingerprints. These bands, however, tended to disappear upon dilution of the DNA stocks. Duplicate assays always corroborated the primary results, as long as DNA contents during PCR was not changed. No differences between the banding patterns were observed when new primer batches were used. Also the fact that strains deriving from a single location in an individual patient gave rise to identical fingerprints is an indication of the reliability of this genotypic procedure.

PCR fingerprinting during surveillance period I: All 99 isolates collected during surveillance period I were subjected to two PCR assays. DNA amplification was carried out either using the single repeat motif ERIC1 (1246) primer or a combination of arbitrary primer BG2 (1236) and the penicillin binding protein gene specific *mec*A2 primer (935) (Figure 1). By combining the results of the different primer tests, 22 different *S. aureus* types were identified among paediatric patients, defined by a two letter code (Table 1A, individuals 1 to 48). Only genotypes A/A, B/B and B/D occurred relatively frequently. Nine individuals were colonised by more than one *S. aureus* strain: multiple colonisations occur in over 18% of all patients. From seven patients serial isolates were obtained. Generally, serial isolates from one particular location were genetically identical. Beside the 99 isolates, 13 were obtained from 10 staff members, working in the same paediatric wards. Two employees were colonised with *S. aureus* type A/S, two with type B/R and three with types J/S, A/Q and K/C, respectively. None of the patients were infected with any of these four types of *S. aureus*. An eighth employee was colonised with type C/C, which was also isolated from three patients, whereas the remaining two employees were found to harbour genotype B/P, which again was found in a singe patient. Patient to staff transfer or vice versa could not be confirmed due to the small numbers of isolates and imprecise contact information (results not shown).

PCR fingerprinting during surveillance period II: At the end of 1992 (October-November) several patients admitted to the paediatric neonatal ward developed skin blisters caused by *S. aureus*. The children were born in the hospital assisted by a constant group of midwives, but various nurses and obstetricians. Initially, 20 strains isolated from two children with infected blisters and five children without symptoms (patients A-G, Table 1B) but admitted to the same ward were analyzed by PCR fingerprinting using three different primer sets (*mec*A2/BG2, ERIC1 and ERIC2) (for examples Figure 2). The genotypes of the bacterial isolates are indicated by a three letter code. Genotypes AAA and BBB are most frequent. Strains of *S. aureus* from five patients present in the neonatology ward during a somewhat later period (patients H-L, Table 1B), were analyzed using a single PCR assay only. The genotypic types are clearly different from those found earlier. No *S. aureus* strains could be isolated from the midwives possibly because screening took place 2 months after the outbreak.

Table IB shows the results of PCR fingerprinting on DNA from *S. aureus* isolated from 6 individuals attending the out-patient clinic (patients I-VI), using three different primer sets (assay 3, 4 and 5). Five different genotypes were found, that did not occur in the hospitalised group. Table 1B also shows the genotypic results of screening an additional group of 21 patients from the outclinic department with the ERIC2 PCR only (patients VIII through XXVII, genotype varies from A to X). In Figure 2C the PCR fingerprints obtained with primer 1220 are shown for the 20 strains isolated from the seven children in the neonatal ward (patients A to G) and for the 6 patients attending the out-patient clinic (I-VI).


A
1.0
0.5

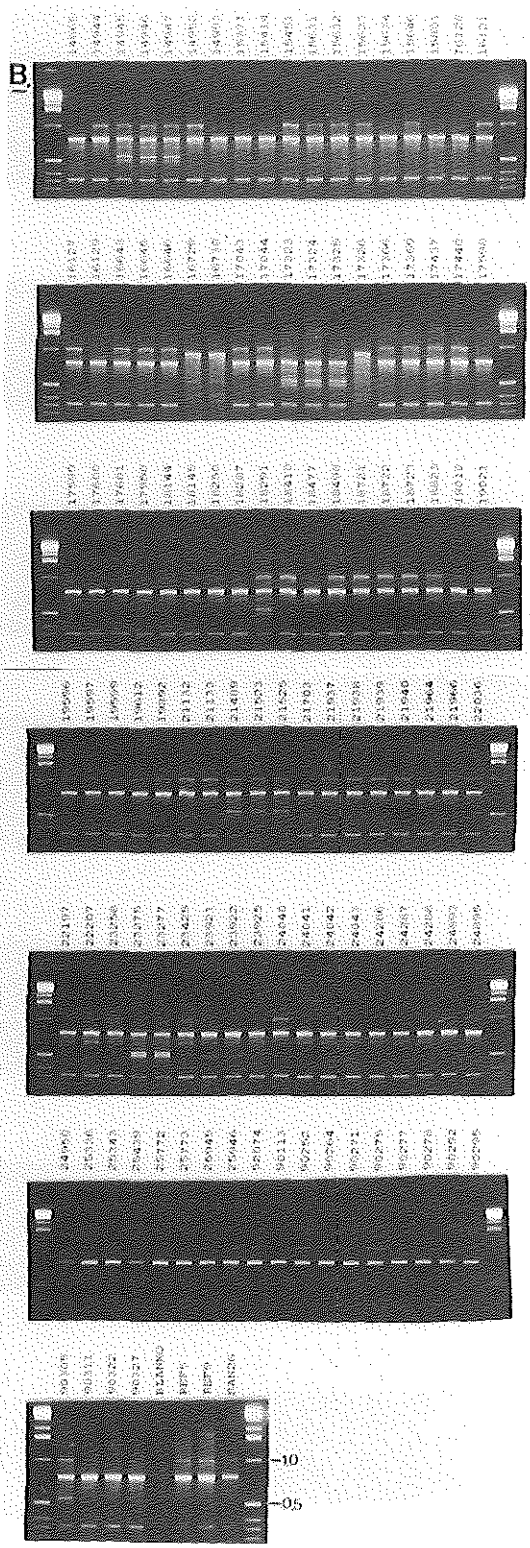

B.
1.0
0.5

Figure 1 PCR fingerprinting of *S. aureus* isolates collected during surveillance period I (July-November 1991) in two pediatric wards of Reinier de Graaf Gasthuis, Delft. Two different PCR assays have been applied; panel A displays the results of DNA amplification by primer combination 935/1236 (mecA2/BG2) whereas panel B displays the results obtained using primer 1246 (ERIC1). Sample numbering correlates with patients and isolates described in Table 1, which also describes the different banding patterns. The two outer lanes contain molecular length markers (1 kB ladder, Gibco/BRL). In the lower panel the length of two of the DNA fragments is indicated in kilobases on the right. The same panel displays the results of a negative control experiment (Blanko) and the results obtained with three unrelated methicillin-resistant *S. aureus* strains (MRSA) isolates (REF5, REF9, SAN26). Numbers starting with 90 (two lower panels) indicate medical personnel.

When all strains collected during surveillance period I are compared, several common genotypes are encountered. These are encoded A/A (n=19), B/D (n=7) and B/B (n=9). It appears that fluctuation in the number of these most frequently encountered genotypes keeps step with variation in the overall number of *S. aureus* isolates (Figure 3). This implies that the more abundant isolates are not epidemic, but occurring more generally in a random population of *S. aureus* strains. On the other hand, when the strains collected in period II are compared, a possible increase in types A and C among paediatric strains as compared to out-patient isolates is observed (Table 1B). In contrast to that observed for surveillance period I, some of the paediatric strains seem to be genetically identical. This clustering may be indicative for a genuine epidemic going on.

Comparison of PCR fingerprints is rather complex. Identification of isolates by specific hybridisation may be more simple. The gel shown in Figure 4 was hybridised with a radiolabeled 850 bp PCR product, indicated by the arrow. The resulting autoradiograph is shown in Figure 4B. The probe detects clonally related organisms, since the PCR fingerprints of the strains reacting with the probe are not completely identical. The relevance of this finding is discussed below.

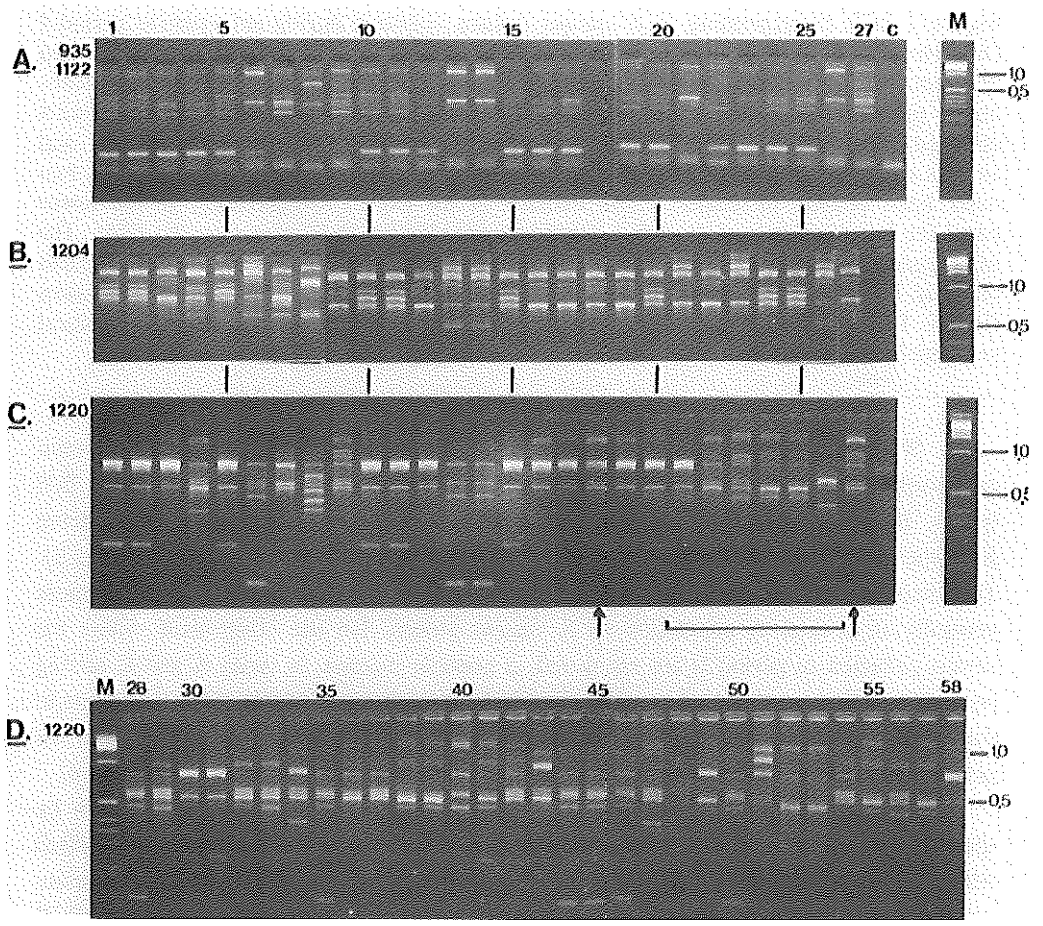


Figure 2 PCR fingerprinting of *S. aureus* isolates collected during surveillance period II (October-November 1992; Table I) from patients in the pediatric department of Reinier de Graaf Gasthuis and from the out-clinic patient population. Three different PCR assays have been applied for a subset of isolates. The upper panels A, B and C display results of experiments using the primers or (combinations thereof) 936/1122 (*mec*A,2/BG2), 1204 (ERIC1) and 1229 (ERIC2), respectively. These three panels show results from the isolates mentioned in the left column of Table 1B (patients A through G, isolates 217121 through 21995) which are numbered from 1 through 20. Molecular length markers are shown. Lanes marked C display the results of negative controls, the arrows indicate PCR results obtained with the same DNA preparation (duplo). Lanes 21-26 (boxed under panel C) display results obtained with DNA isolated from bacterial strains isolated from out-clinic patients (patients I through VI, right column Table 1B) from the same period. Panel D displays the PCR fingerprinting results for additional pediatric isolates. The results presented in lanes 33, 34 and 52 to 57 correspond to strains deriving from patients H through L (in the same order as shown at the bottom of the left column in Table 1B). A larger panel of out-clinic patient isolates is also shown. All isolates were only screened using primer 1220 (ERIC2). Samples shown in panel A have been electrophoresed under slightly different conditions. Numbering of the lanes is mentioned in Table 1B as well.

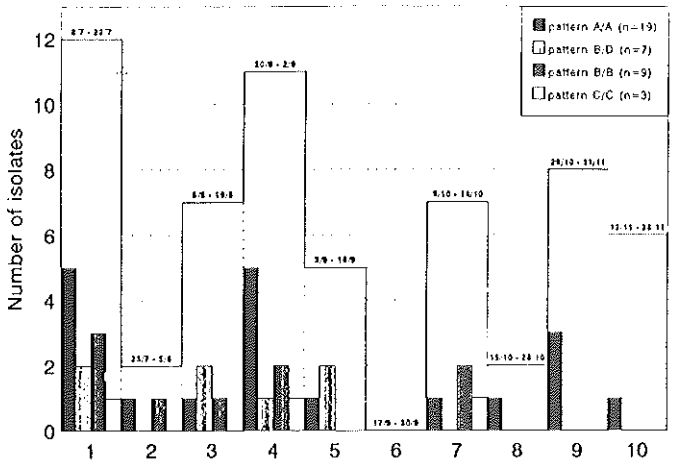


Figure 3 Frequence of occurrence of the 4 major *S. aureus* genotypes during the first 20-week surveillance period. The number of isolates, encoded by a particular shade of grey, is given on the vertical axis, the overall number of strains characterised during a 2-week interval is displayed in white and the horizontal scale displays time in fortnights. Precise dates (1991) are indicated above the major columns. Data presented in this figure can be traced in Table 1 as well. Multiple, but identical strains from a single patient are scored as being one isolate.

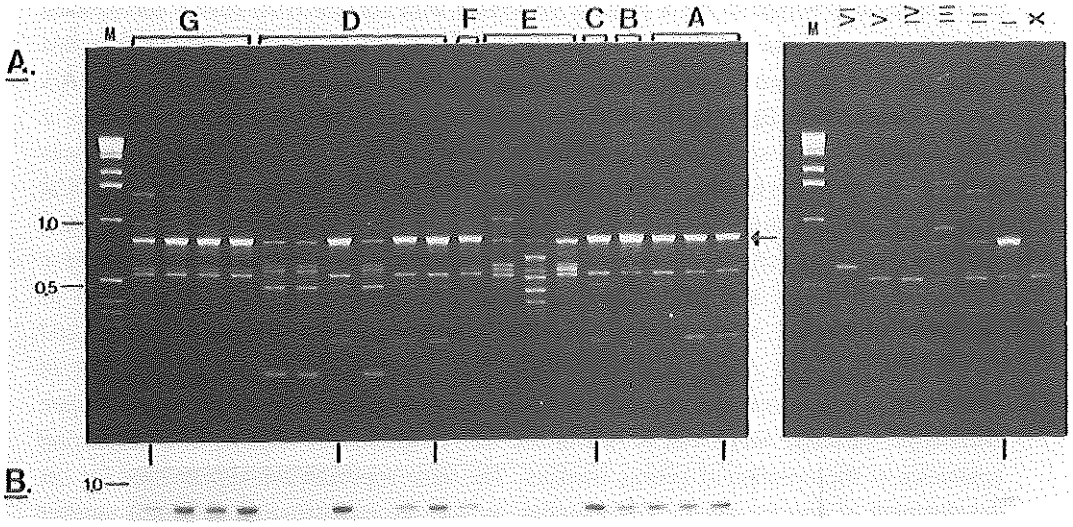


Figure 4 The use of PCR fingerprinting and DNA probing in the recognition of *S. aureus* isolates. Panel A displays PCR fingerprinting results obtained with primer 1220. Isolates have been grouped according to patient (A to G, Table 1). Lanes I to VI show isolates obtained from out-clinic patients. These isolates date from the same period (II). Panel B shows the autoradiograph obtained upon hybridisation of the Southern blot generated from the gel displayed in panel A with the DNA fragment that is indicated by an arrow in that same panel. Only a subset of the *S. aureus* fingerprints contain homologous DNA fragments, indicating the isolate specificity of the DNA probe. Whereas the probe reacts with 68% of the pediatric strains, only 14% of the out-clinic strains show hybridisation.

DISCUSSION

During a genuine nosocomial epidemic the bacterial isolates from different patients should be closely related. This putative homogeneity of strains can best be measured by DNA mediated analyses. Amplification of variable DNA regions provides a sensitive and versatile method for the genetic identification of large collections of microorganisms (1, 3, 7, 10, 11, 12). In the present paper PCR assays monitoring genetic variability have been applied to study the dynamics of *S. aureus* infections. In the paediatric patients presented in this study 18% harboured more than one genotypically distinguishable bacterial strain. We also found that once a strain is settled in a certain anatomical location it will be detectable in that location over a longer period of time. It is interesting to note that PCR assays that were initially developed for typing methicillin-resistant *S. aureus* (1, 10) detected a relatively high degree of genetic variability in MSSA as well. This is probably due to the low strigency of annealing conditions applied during amplification, which renders the precise nucleotide orders of the primers of lesser importance.

There appears to be a strong variation in the absolute number of *S. aureus* infections encountered during defined and consecutive periods of time in a single hospital ward. Apparently, the fluctuation in the occurrence of the most frequently observed genotypes keeps step with variation in overall numbers. As a result it was impossible to link a specific genotype to the 4 patients presenting with conjunctivitis during surveillance period I. Results obtained from the second period are somewhat different. During this period 12 patients were screened, three patients of which were colonised with the AAA type, and three with BBB. In the outclinic population genotype A was only found once in 27 individuals. This suggests an epidemic in the neonatal ward. Additional proof was obtained by Southern blotting analysis. Using the most prominent band of the 1220 PCR fingerprint as DNA probe, hybridisation was found with A and B genotypes, indicating a close genetic relationship between these genotypes and maybe a recent common ancestor (Figure 4). It seems that PCR fingerprinting derived DNA fragments can be used to identify a subset of *S. aureus* strains. Obviously a larger number of DNA fragments could be used as such and panels of strain-specific DNA probes can be developed (work in progress). These panels can be used to establish genetic relatedness or to investigate phenotypic variants, which can be useful for the recognition of ongoing or re-occurring epidemics or the isolation of genetic markers. The same approach can also be used for the development of species-specific DNA probes, as was recently demonstrated for a number of species belonging to the genus *Campylobacter* (6). Apparently, DNA probes aiming at various taxonomic levels can be isolated relatively easily as a sideproduct of PCR fingerprinting.

The practical means for typing *S. aureus* are rather limited. Phagetyping is often unsatisfactory, due to practical complexity and its inability to type all isolates. Phagetyping of several of the isolates described in this paper did not yield useful information (results not shown), which is in contrast with previous findings for methicillin-resistant *S.*

aureus strains (1). The reason for this observation is not clear, a more extensive comparison is required. The application of DNA ribotyping is hampered by laborious procedures and a low discriminative power. In a recent study (9) a detailed comparison between several typing schemes for *S. aureus* was described: capsular typing, zymotyping, phagetyping and genome typing. Maximum resolution appeared to be in the order of 40%. We found that a by single PCR assay 25 different genotypes can be defined in a random group of 58 *S. aureus* isolates gathered during surveillance period II (resolution of 43%). Increasing the number of primers will lead to an increase in genotypes as we showed with the strains isolated during surveillance period I. Recently, the value of the PCR fingerprinting procedure used here was underscored by direct comparison of PCR results with the detection of restriction fragment length polymorphisms (RFLP, see ref. 10). Results obtained by fingerprinting correlated well with those of the macrorestriction analyses. Findings like these strongly support the data presented in this report.

Identification by PCR fingerprinting can be used to monitor colonisation and infection as shown in the present study with a resolution that exceeds that of the more conventional typing assays. Obviously, the same strategy can also be used for studying epidemics caused by other nosocomially occurring bacterial pathogens.

REFERENCES

1. **Belkum A. van, R. Bax, P. Peerbooms, W.H.F. Goessens, N. van Leeuwen and W.G.V. Quint** (1993) Comparison of phagetyping and DNA fingerprinting by PCR for discrimination of methicillin-resistant *Staphylococcus aureus* strains. J. Clin. Microbiol. **31**, 798-803.
2. **Boom R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-van Dillen and J. van der Noordaa** (1990) Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28**: 495-503.
3. **Bruin F.J. de** (1992) Use of repetitive sequences and the PCR to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Env. Microbiol. **58**: 2180-2187.
4. **Erlich H.A., D. Gelfand and J.J. Sninsky** (1991) Recent advances in the polymerase chain reaction. Science **252**: 1643-1651.
5. **Feinberg A. and B. Vogelstein** (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**: 6-13.
6. **Giesendorf B., A. van Belkum, A. Koeken, H. Stegeman, M. Henkens, J. van der Plas, H. Goossens, H.G.M. Niesters and W.G.V. Quint** (1993) Development of species-specific DNA probes for *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari* by Polymerase Chain Reaction fingerprinting. J. Clin. Microbiol. **31**, 1541-1546.
7. **Mazurier S., A. van de Giessen, K. Heuvelman and K. Wernars** (1992) RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Letters in Appl. Microbiol. **14**: 260-262.

8. **Sambrook J., F. Fritsch and T.E. Maniatis** (1989) Molecular cloning. A laboratory manual Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
9. **Schlichting, C., C. Branger, J.M. Fournier et al** (1993) Typing of *S. aureus* by pulsed field gel electrophoresis, zymatology, capsular typing and phagetyping: resolution of clonal relationships. J. Clin. Microbiol. **31**: 227-232.
10. **Struelens, M., R. Bax, A. Deplano, W. Quint and A. van Belkum** (1993) Concordant clonal delineation of methicillin-resistant *Staphylococcus aureus* by macrorestriction analysis on PCR genetic fingerprinting. J. Clin. Microbiol. 31, 1964-1970.
11. **Versalovic J., T. Koeuth and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19**: 6823-6831.
12. **Welsh J. and M. McClelland** (1991) Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. Nucleic Acids Res. **19**: 5275-5279.

Adapted from: J. Clin. Microbiol. 1993;31:798-803

CHAPTER X

COMPARISON OF PHAGETYPING AND DNA FINGERPRINTING BY PCR FOR DISCRIMINATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS

Alex van Belkum, René Bax[1], Paul Peerbooms[2], Wil H.F. Goessens[3], Nan van Leeuwen[4] and Wim G.V. Quint[1]

[1]Diagnostic Centre SSDZ, P.O. Box 5010, 2600 GA Delft, The Netherlands. [2]Municipal Health Service Amsterdam, Nieuwe Achtergracht 100, 1018 WT Amsterdam, The Netherlands. [3]Academic Hospital Dijkzigt, Erasmus University Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands. [4]National Institute of Public Health and Environmental Protection, P.O. Box 1, 8720 BA Bilthoven, The Netherlands.

SUMMARY

A typing procedure for methicillin-resistant *Staphylococcus aureus* (MRSA) based on the (PCR) amplification of both *mecA* sequences and variable DNA sequences as present in the prokaryote genome has been developed. Two primers based on the sequences of DNA repeats as discovered in gram-negative members of the family of *Enterobacteriaceae* allow detection of variable regions in the genome of a gram-positive bacterium such as *Staphylococcus aureus*, as does a newly described arbitrary primer. This procedure, enabling the detection of 23 different genotypes in a collection of 48 MRSA isolates, was validated by comparisons with phage typing studies. It appeared that within the same group of isolates only 13 different phagovars could be identified. Combination of the results from both phage typing and genotyping allowed the discrimination of 34 out of 48 isolates. However, depending on the primer-variable complexity of the PCR fingerprints, which could also be modulated by combination of PCR primers, clear homologies between the groups defined either by phage typing or fingerprinting were observed. An analysis of an MRSA outbreak in a geriatric institution showed a collection of genetically homogeneous isolates. In agreement with phage typing, PCR fingerprinting revealed the identical natures of the MRSA strains isolated from all patients.

INTRODUCTION

Several biochemical and molecular biological parameters can be used to distinguish isolates of a given genus or species. Many procedures for typing of methicillin resistant *Staphylococcus aureus* (MRSA) have been developed (2, 6, 14). Besides the traditional phage typing method for *S. aureus*, several alternative techniques have been applied in comparative, epidemiological studies. Among these are serotyping (13), capsular typing (18), electrophoretic analysis of protein content (5), pulsed-field gel electrophoresis (10), restriction fragment length polymorphism (RFLP) analyses (10), and ribotyping (14). In addition to epidemiological studies on clinical isolates, attention has been focused on the development of adequate detection assays for MRSA. The polymerase chain reaction

(PCR) has proven to be an adequate means for the detection and typing of a multitude of microorganisms (8, 9, 11, 16). As a consequence, several PCR and probe assays specific for the *mecA* gene of *S. aureus*, encoding the penicillin-binding protein 2a, have been evaluated (1, 7, 12, 19, 20). PCR at present allows sensitive detection of MRSA in clinical specimens (12). For typing of microorganisms in particular, random amplified polymorphic DNA (RAPD) analysis is more and more frequently used in order to discriminate between individuals or isolates of a given species (4, 23, 24). As an alternative to the arbitrary RAPD analysis it is feasible to amplify known polymorphic regions within DNA molecules in a way which gives rise to variable DNA fingerprints. Repeated DNA motifs are especially amenable to this approach. PCR fingerprinting using primers aiming at, for instance, eukaryotic simple sequences or telomeric repeats (21) or prokaryotic repetitive extragenic palindromes (REP) and enterobacterial repetitive intergenic consensus sequences (ERIC) (22) proves to be of value in the identification of genetically similar but not identical organisms or isolates of a given species.

In this study, traditional phage typing of MRSA was compared with newly developed PCR fingerprinting procedures aiming both at the *mecA* gene, as a determinant for methicillin resistance, and at variable DNA sequences. This comparison allows evaluation of both procedures, and their practical applicability is discussed.

MATERIALS AND METHODS

Bacterial strains: Forty-eight randomly isolated clinical strains of MRSA were provided by the Laboratory for Medical Microbiology of the Academic Hospital Dijkzigt in Rotterdam, the Netherlands. These strains were identified by standardised microbiological procedures. All strains were identified as *S. aureus* on the basis of colony morphology, colour, and a positive catalase and coagulase test. Phenotypic methicillin resistance was determined by the disk diffusion assay as described. Briefly, isolated colonies from an overnight plate are suspended in saline to a turbidity of 0.5 McFarland standard. To inoculate the Mueller-Hinton agar plates (Oxoid CM 337), a cotton swab is dipped into the standardised suspension and streaked in three directions over the entire plate surface to obtain a uniform inoculum. Subsequently, a disk containing 5 μg of methicillin (Oxoid) is applied. Plates are inverted and incubated for the full 24 h at a temperature of 30°C. Plates are examined and the diameters of the inhibition zone are measured by a template. A diameter of smaller than 17 mm is regarded as resistant or in the case of growth of separate colonies in the inhibition zone strains were also regarded as mthicillin resistant. The Municipal Health Service of Amsterdam provided a collection of MRSA isolates from a single outbreak from a geriatric nursing house and harbouring multiple isolates from individual patients. Variations were in body sites from which the sample was taken and the date of sampling. Also, a single isolate displaying a phage type, which strongly deviated from the ones obtained for the Dijkzigt isolates was provided. This strain was

analyzed together with those of the outbreak. All strains were grown as monocultures in Brain Heart Infusion broth and incubated in a rotary shaker at 37°C for 18 h.

DNA isolation: Since isolation of *S. aureus* is hampered by the rigidity of the bacterial cell wall, lysostaphin treatment is generally included in the preparation of lysates. Combinations of several extraction procedures were tested for efficacy of DNA isolation. A sample of 500 μl of the cultures was preincubated either with 15 μg lysostaphin or 25 μg proteinase K and 10 μl 10% sodium dodecyl sulphate (SDS) to digest the bacterial cell wall. Incubation took place for 1 h at 37°C. The subsequent isolation and purification of DNA was performed either with 4M guanidinium-isothiocyanate and Celite according to previously described procedures (3) or by phenol extraction followed by ethanol precipitation (17). DNA concentrations were estimated by gel electrophoresis through 1% agarose gels in 40 mM Tris-acetate pH 7.8, 2 mM EDTA (TAE), staining with ethidium bromide, and comparison with samples containing a known amount of DNA (17). Proteinase K - SDS treatment, combined with guanidinium isothiocyanate extraction, generated DNA preparations of the highest quality.

Phage typing: Analysis of MRSA phage susceptibility was performed at the Dutch National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands). An international set of typing phages, an additional set of Dutch phages as well as MRSA-specific phages were used for the establishment of the phagovar.

TABLE 1 DNA PRIMERS EVALUATED FOR APPLICABILITY IN PCR-MEDIATED TYPING OF MRSA ISOLATES

primer number	primer sequence (5' to 3')	primer type	literature reference
2650	AATCGGGCTG	arbitrary primer	(4)
961	AATCGGGCNG	arbitrary primer	this paper
2651	CAATCGCCGT	arbitrary primer	(4)
963	CAATCGCCNT	arbitrary primer	this paper
1026	TACATTCGAGGACCCCTAAGTG	arbitrary primer	this paper
1116	TCACGATGCA	arbitrary primer	(24)
934	AAAATCGATGGTAAAGGTTGGC	MecA gene 1	(12)
935	AGTTCTGCAGTACCGGATTTGC	MecA gene 2	(12)
1064	TTGACGAAAATTGAATGGAGAATAGACGTTAAAATGAATC	*S. aureus* dru sequence	(15)
1065	CTAAGTAAAATTGCAGATAAGAGGTAAGTTAAAAGCAGTT	*S. aureus* inverted dru sequence	(15)
1204	GTGAATCCCCAGGAGCTTACAT	ERIC1R	(22)
1014	AAGTAAGTGACTGGGGTGAGCG	ERIC2	(22)
933	$(AGC)_5$	simple sequence motif	(21)

Primers and primer synthesis: Oligodeoxyribonucleotide primers were synthesised on an Applied Biosystems DNA synthesizer (model 381A) and used without any further purification. DNA concentrations were determined by measuring the absorbency at 260 nm on a Uvicam spectrophotometer (model PU 8720). A list of the primers used is presented in Table 1.

PCR: The reaction mixtures consisted of 10 mM Tris.HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100. Deoxyribonucleotide triphosphates

TABLE 2 COMPARISON OF PHAGE TYPING AND PCR FINGERPRINTING OF 48 MRSA ISOLATES

phagovar	strains nr.	935 1026	1204	1014	1014 1026	phagovar	strain nr.	935 1026	1204	1014	1014 1026
E1	10	A	I	H	O	E var.	28	B	I	K	V
	19	A	I	H	P	2638	33	A	II	H	P
	20	A	I	H	O		34	A	II	H	P
	22	A	I	H	Q		43	A	I	H	T
	37	A	I	H	R		45	A	I	H	U
	42	A	I	H	Q						
	49	A	I	H	S	E var.	13	A	I	H	Q
	50	A	I	H	Q	2623	25	A	I	H	P
							29	A	I	I	O
E2	1	A	I	H	S		31	A	I	H	P
	7	A	I	I	O						
	11	A	I	H	S	E var.	14	C	II	L	P
	15	A	I	H	R	2624	26	C	II	L	P
	16	A	I	H	Q		30	C	II	L	V
	39	A	I	J	S		35	C	II	L	V
	40	A	I	H	O		41	C	II	L	T
E3	8	A	I	I	O	Evar.2627	17	A	I	M	P
E var.	2	A	I	H	T	Evar.2631	21	D	IV	J	W
2612	4	A	I	H	T						
	5	A	I	H	T	Evar.2637	27	A	II	L	P
	12	A	I	H	T						
	18	A	I	H	U	Evar.2616	6	C	II	L	X
	24	A	I	H	U						
	32	A	I	H	P	III-49	9	E	III	J	T
	44	A	I	H	T		23	F	III	J	T
	48	A	I	K	T						
						n	36	G	IV	J	R
							46	G	IV	J	Q
							47	G	IV	J	R

Note: This table gives a combined survey of phage typing (13 types, E1 to n) and genotyping (fingerprint A to X and I to IV). On top the various primers (or combinations thereof) as applied in the PCR tests are indicated. Genotypic characteristics correspond to those shown in the various figures. Strains were identified by their laboratory number (1 to 50). The total number of strains was 48. The number of variant genotypes (combination of four PCR tests) is 23. Combining DNA and phage typing allows discrimination of 34 out of 48 isolates.

were used at a final concentration of 0.2 mM. For each reaction, 0.5 units of Taq DNA polymerase (Sphaero-Q, Leiden, The Netherlands) was added. PCR conditions consisted of 40 cycles of consecutive denaturation, annealing, and DNA chain extension (1 min 94°C, 1 min 25°C, 2 min 74°C) in a Biomed thermocycler (Theres, Germany, model 60). Southern blots and probe hybridisation were performed as described before (12).

RESULTS

Conventional *S. aureus* strain typing: For 48 MRSA strains, the presence of the methicillin resistance gene complex was confirmed by *mecA*-specific PCR followed by Southern blot hybridisation of the PCR products (data not shown) (12). Phage typing identified thirteen phagovars of MRSAs. The number of strains belonging to each phagovar varied from 1 to 9. Most of the strains used could not be typed by application

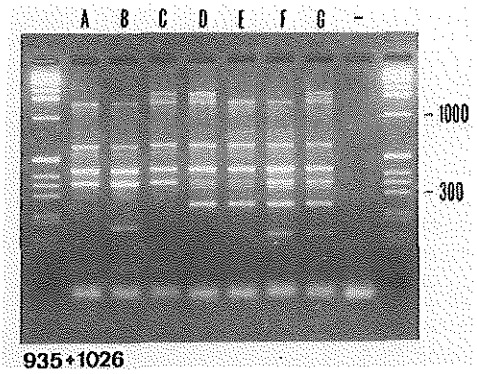


Figure 1 PCR-mediated DNA typing of MRSA strains. Lanes A to G show the various banding patterns among a collection of 48 MRSA strains. Primers 935 and 1026 were used (Table 1). "-", negative control. The length of two of the molecular size markers (1-kb ladder, GIBCO BRL) is indicated on the right, in base pairs.

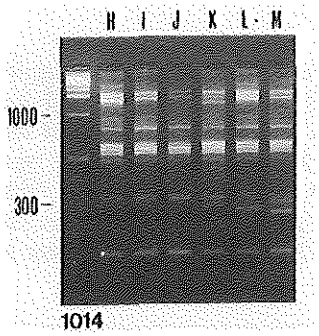


Figure 2 PCR-mediated DNA typing of MRSA strains. With primer 1014 (Table 1), six different genotypes, marked H to M, were identified in a group of 48 MRSA strains. The length of two of the molecular size markers (1-kb ladder, GIBCO BRL) is indicated on the left, in base pairs.

of the international phage sets. Phages of the international group III showed some reactivity, only with isolates 9 and 23. For all other strains, the use of a collection of Dutch MRSA-specific phages led to the discrimination as shown in Table 2. The phages used (and descriptions of those phages) are available on request.

PCR fingerprinting. An increase in the ratio between genomic DNA and PCR primers resulted in an overall synthesis of longer amplimers; smaller fragments tended to disappear from the DNA banding patterns. Probably, the higher the primer concentration, the more primers may anneal to less specific target sequences, causing more and smaller PCR fragments to be generated. The best interpretation of the DNA fingerprint results was achieved with 5 ng of DNA and a total of 100 pmol of primers (either single or in combination) per reaction (data not shown). Standardisation of the PCR in this way also rendered the assays highly reproducible: some small differences in fingerprinting patterns could be demonstrated repeatedly. The 48 bacterial strains were subjected to PCRs with different primers or primer combinations. Among those were arbitrary primers 933, 961,

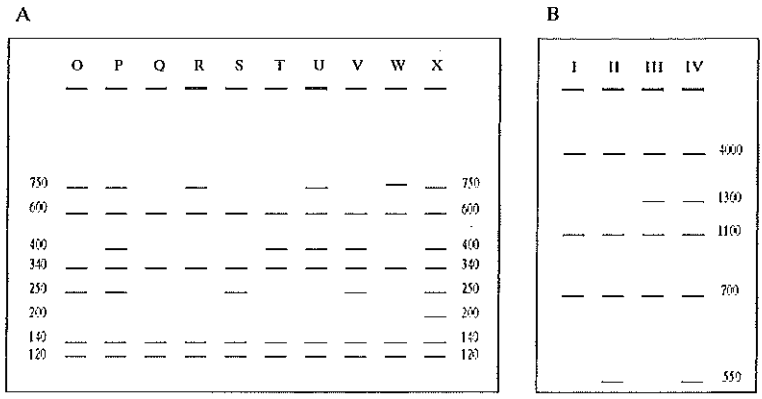


Figure 3 Schematic presentation of the DNA banding patterns obtained by PCR fingerprinting with a combination of primers 1014/1026 (A) and primer 1204 (B) involving DNA from MRSA strains. Displayed are genotypes O to X and I to IV; lengths of the DNA fragments are indicated. Note that the largest visible DNA fragment in the W type is slightly longer than similar fragments present in types O, P, R, U and X (A). With primer 1204, longer DNA fragments were synthesised. The lengths of the fragments, as deduced from a comparison with a co-electrophoresed GIBCO BRL 1-kb length marker is indicated in the margins, in basepairs.

963, 1116, 2650 and 2651 (4); primer *MecA*-1 (12), two primers constituting the *dru*consensus sequence at the *mec*-locus of MRSA DNA (15; Table 1). The *dru*-sequences were synthesised in both the 5' and 3' directions. However, neither the primers individually nor combinations of these primers produced satisfying results; either the patterns were identical for most strains or there were no visible DNA banding patterns (data not shown). The identities of fingerprints obtained with a certain primer or a primer combination imply, alternatively, that the PCR assay itself could be used for confirmation of MRSA. The banding pattern obtained in this way identifies the species as such. Distinct banding patterns of PCR fragments were obtained with primers 935, 1014, 1026 and 1204 either alone or in combination. Subjection of DNA from the 48 different MRSA strains to PCR with primers 935 and 1026 revealed seven different banding patterns (Figure 1). PCR analysis with primer 1014 revealed six different banding patterns within this group (Figure 2). When the 48 MRSA strains were subjected to PCR by primers 1014 and 1026, ten different banding patterns arose (Figure 3A). PCR using primer 1204 led to the discovery of another four fingerprint types (Figure 3B). Application of the latter primer resulted in the PCR-mediated synthesis of relatively long DNA fragments. A comparison

of the DNA banding patterns seen in Figure 1-3 reveals that a certain size homology exists between DNA fragments found in the various genotypes identified by these three PCR approaches. PCR using a single primer or a combination of two primers allows the detection of only a limited number of genotypes. Combination of the individual DNA fingerprints obtained with various primers or combinations, however, led to the discrimination of 23 different genotypes in the collection of 48 MRSA strains.

Correlation between DNA fingerprinting and phage typing: The PCR fingerprinting results were compared with the results for typing with MRSA-specific bacteriophages (Table 2). Fingerprinting with the combination of primers 935 and 1026 showed a considerable overlap with the phage typing. Phagovars E1, E2 and E3 could be grouped as having a single banding pattern (A). Variations of phagovar E could be classified in groups with common characteristics. Some of these groups show the same banding pattern as the former (E2612, E2623, E2627, E2637 and E2638, except strain 28). The other phage type variants (E2624, E2631 and E2616) and phagovar n all displayed unique fingerprints with the combination of primers 935 and 1026.

When primer 1014 was used, several of the aforementioned groups could be differentiated into more subtypes. E2 phagovars, for instance, could be divided into three genotypically different groups (H, I, and J). On the other hand, phagovars E1 and E2624 still appeared to be genetically homogeneous groups. In the E2638 group, one of the isolates (number 28) showed a deviating genotype with either primer 1014 or primers 935 and 1026, which in itself is consistent with nucleotide sequence changes taken place in this particular isolate only. Application of primer 1204 enabled a further discrimination of individual genotypes in the E2638 group. Strains 6, 17, 21 and 27 are single isolates belonging to unique phagovars. The PCR fingerprints for these isolates are, in general, also not found in the other phagovar groups. From Table 2 it can be concluded that, depending on the PCR primers, lysotypically determined groups of strains can be either grouped in a similar way (see primer combination 935 and 1026 and 1014 for phagovar E1) or can be divided into various genetically different subgroups (see primer combination 1014 and 1026 for phagovar E2). This implies that, in principle, this PCR approach allows distinction of the same groups of MRSA isolates, and, in addition to that, it also enables the division of these groups into smaller units. This is of importance in epidemiological analyses, since the higher the discriminative power is, the lower the chances that independently isolated MRSA strains will still appear to be identical. On the other hand, it should not be forgotten that extremely variable DNA loci are not suited for application in epidemiology, since in this case the resolution of the procedure may be too profound.

Analysis of an MRSA epidemic: The genetic homogeneity of strains isolated during an MRSA epidemic in a geriatric nursing home was studied by the PCR fingerprinting procedure. As a control, the additional MRSA isolate having a unique phage type, was included (see Materials and Methods). With primers 935 and 1026 in the PCR

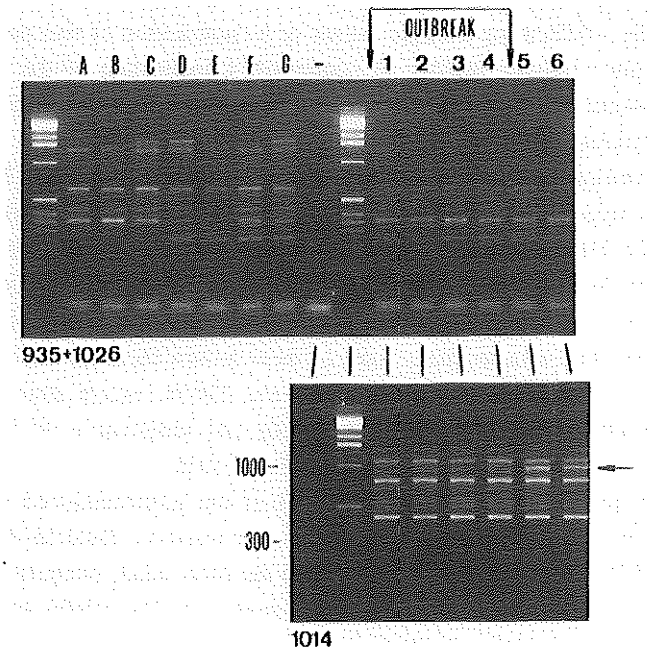


Figure 4 DNA typing of MRSA strains from diverse sources. Lanes A to G display results similar to those in Figure 1. Shown is the diversity in DNA fingerprints observed in a group of 48 MRSA isolates. Isolates 1 to 6 represent strains either from an MRSA outbreak in a geriatric nursing home (1 to 4) or from another patient (5 and 6). The outbreak samples, of which 24 different isolates were available, appear to be genetically homogeneous, as indicated by PCR-mediated genotyping with both primer 1014 and primer combination 935-1026. The latter combination does not enable discrimination of samples in lanes 5 and 6 or 1 to 4. Primer 1014, however, clearly differentiates the large group, which showed identical banding patterns, from the strain not isolated in the geriatric hospital (see the arrow on the right, indicating an additional band in lanes 5 and 6). The lengths of the molecular size markers (1-kb ladder, GIBCO BRL) are indicated on the left of the lower panel, in base pairs.

genotyping assay, no discrimination could be made between outbreak related strains from the home for the elderly and the unrelated strain (Figure 4, lanes 5-6). All strains displayed genotype F (Figure 4, upper panel). Primer 1204 again revealed the genetic homogeneity of all outbreak strains. The DNA banding pattern obtained was identical to pattern IV shown in Figure 3B. Outbreak strains and an unrelated isolate could not be discriminated as well. Interestingly, with primer 1014, outbreak strains and the reference isolate could be discriminated quite clearly. In comparison to genotypes H to M found in the collection of 48 MRSAs, two new PCR fingerprints were obtained. All isolates from

the outbreak were genetically homologous and the independent isolates (Figure 4, lower panel, lane 5 and 6) showed a DNA banding pattern which differed from the fingerprints of the isolates from the home for the elderly (Figure 4, lower panel: the arrow indicates an additional band in the reference strains). In conclusion, the latter observations substantiate the potential applicability of the PCR-mediated genotyping in epidemiological analyses. Apparently, it is possible to define groups of closely related genetic composition, which is of utmost importance in comparative analyses of the spreading of certain organisms.

DISCUSSION

Until recently, the method best suited for obtaining direct insight in the variability of genomes was the determination of restriction fragment length polymorphisms (RFLPs). However, this method is complicated and time-consuming and is therefore not very well suited for use in a routine diagnostic setting. The development of PCR methods enabling the identification of complex DNA molecules provides a relatively rapid and simple method to achieve similar goals. Frequently occurring nosocomial epidemics are multiple infections due to acquisition of methicillin-resistant *Staphylococcus aureus* strains. The present method of choice for sorting out these epidemics is the typing with MRSA-specific bacteriophages. This procedure is not without pitfalls. However, whereas phage typing may be hampered by phenotypical variation (e.g. growth stage, culture medium, etc.), the PCR typing approach described here is less vulnerable to environmental influences.

On the other hand, the results show that successful application of PCR fingerprinting depends heavily on the primer choice. Random amplified polymorphic DNA (RAPD) PCR with short arbitrary primers was not successful. Also, the use of simple sequence-resembling primers or primers based on MRSA *dru* repeats did not enable accurate discrimination of MRSA strains. The best resolution was obtained by applying the enterobacterial repeat consensus primers 1014 and 1204 (Figures 2, 3B and 4), a combination of 1204 with an arbitrary primer (1026, Figure 3A), or the combination of the *mecA2* primer with the same arbitrary primer (Figure 1). The presence of ERIC-like sequences has not been demonstrated for gram-positive bacteria as yet. It seems that under less stringent PCR conditions also the ERIC1 and -2 sequences encounter some homologies in the genomes of gram-positive bacteria as well. Combining *mecA* sequence-based primers with an arbitrary primer (Figure 1) illustrates the useful combination of constant methicillin resistance gene sequences and variable DNA sequence motifs. Applying genotyping studies to known and constant regions allows combined identification of several phenotypic and genotypic characteristics of a given microorganism in one assay. Aiming at both *mecA* sequences and variable DNA loci should allow methicillin resistance determination together with genetic identification of MRSA strains. Several of the primers surprisingly produce fingerprinting schemes that overlap with grouping based on

phage typing. Interestingly, several of the bands found in the PCR fingerprints are shared among different genotypes (Figure 1). Probably this still reflects a close relationship among these isolates, which in the end could serve as an additional means of MRSA identification, and, possibly, strain classification. This also requires examination of methicillin-sensitive *S. aureus* strains. In this study, methicillin resistance of the (clinical) isolates was a prerequisite for inclusion in the study population.

Five phagovars occur only once in the MRSA collection studied. PCR fingerprints of four of these strains are unique as well, which may be considered to support the validity of the PCR method. Twenty-three combinations of DNA banding patterns were identified. When these overall fingerprints were combined again with results of the phage typing, it was possible to distinguish 34 subtypes of *S. aureus* in 48 strains (Table 2). It can be imagined that whenever additional primers or primer combinations will be used, the discrimination of strains will proceed, resulting in a personal combinatory fingerprint for each strain. The latter is not useful in the examination of epidemics. It was already noticed that concentrating PCR products by ethanol precipitation allows the visualisation of additional, minor DNA fragments. However, this severely complicates the analysis and for that reason these results were not included in figures and tables. It will be interesting to examine the exact nature of the DNA fragments that are synthesised during PCR.

In conclusion, a PCR-mediated procedure which enables straightforward genetic typing of MRSA isolates and, at the same time, correlates well with established phage typing procedures is described. The applicability in the analysis of MRSA outbreaks is demonstrated. Because of the general character of the primers used, this type of PCR-mediated DNA identification procedures can be applied to a multitude of other microorganisms as well. Present studies on non-MRSA isolates, for instance, confirm this latter suggestion (unpublished data). Comparisons between PCR fingerprinting and the accepted molecular techniques applied in epidemiological analyses will have to be made in order to validate PCR genotyping. However, practical application of this PCR-mediated procedure is feasible only when PCR strategies are simplified and when automated systems for reproducible generation and interpretation of DNA fingerprints are available. Finally, elucidation of the way of spreading of microorganisms in clinical settings as traced by PCR fingerprinting can have a significant impact on the measures to be taken to prevent subsequent infections.

REFERENCES

1. **Archer, G.L. and E. Pennell** (1990) Detection of Methicillin Resistance in Staphylococci by Using a DNA Probe. Antimicrob. Agents Chemother. **34**: 1720-1724.
2. **Blumberg, H.M., D. Rimland, J.A. Kiehlbauch, P.M. Terry, and I.K. Wachsmuth** (1992) Epidemiologic typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphisms of rRNA genes: Elucidation of the

clonal nature of a group of bacteriophage-nontypeable, ciprofloxacin-resistant, methicillin-susceptible *S. aureus* isolates. J. Clin. Microbiol. **30**: 362-369.

3. **Boom, R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-van Dillen, and J. van der Noordaa** (1990) Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28**: 495-503.
4. **Caetano-Annollés, G., B.J. Bassam, and P.M. Gresshoff** (1991) DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Biotechnology **9**: 553-557.
5. **Costas, M., B.D. Cookson, H.G. Talsania and R.J. Owen** (1989) Numerical analysis of electrophoretic protein patterns of methicillin-resistant strains of *S. aureus*. J. Clin. Microbiol. **27**: 2579-2581.
6. **Goh, S-H., S.K. Byrne, J.L. Zhang, and A.W. Chow** (1992) Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphisms. J. Clin. Microbiol. **30**: 1642-1645.
7. **Hackbarth, C.J. and H.F. Chambers** (1989) Methicillin-Resistant Staphylococci: Genetics and mechanisms of resistance. Antimicrob. Agents Chemother. **33**: 991-994.
8. **Hermans, P.W.M., A.R.J. Schuitema, D. van Soolingen, G.P.H.J. Versteynen, E.M. Bik, J.E.R. Thole, A.H.J. Kolk and J.D.A. van Embden** (1990) Specific detection of *Mycobacterium tuberculosis* complex strains by Polymerase Chain Reaction. J. Clin. Microbiol. **28**: 1204-1213.
9. **Jiwa, N.M., G.W. van Gemert, A.K. Raap, F.M. van de Rijke, A. Mulder, P.F. Lens, M.M.M. Salimans, F.E. Zwaan, W. van Dorp and M. van der Ploeg** (1989) Rapid detection of human cytomegalovirus DNA in peripheral blood leukocytes of viremic transplant recipients by the polymerase chain reaction. Transplantation **48**: 72-76.
10. **Jordens, J.Z. and L.M.C. Hall** (1988) Characterisation of methicillin-resistant *Staphylococcus aureus* isolates by restriction endonuclease digestion of chromosomal DNA. J. Med. Microbiol. **27**: 117-123.
11. **Melchers, W.J.G., A.C.J. van de Brule, J.M.M. Walboomers, M.A. de Bruin, M.A. Burger, N. Burger, P. Herbrink, C.J.L.M. Meyer, J. Lindeman, and W.G.V. Quint** (1989) Increased detection rate of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared with modified FISH and Southern blot analysis. J. Med. Vir. **27**: 329-335.
12. **Murakami, K., W. Minamide, W. Wada, E. Nakamura, H. Teraoka, and S. Watanabe** (1991) Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. J. Clin. Microbiol. **29**: 2240-2244.
13. **Oeding, P** (1957) Serological typing of staphylococci. Acta Pathol. Microbiol. Scand. **93**: 356-363.
14. **Prevost, G., B. Jaulhac, and Y. Piemont** (1992) DNA fingerprinting by pulsed-field gel electrophoresis is more effective than ribotyping in distinguishing among methicillin-resistant *Staphylococcus aureus* isolates. J. Clin. Microbiol. **30**: 967-973.
15. **Ryffel, C., R. Bucher, F.H. Kayser and B. Berger-Bächi** (1991) The *Staphylococcus aureus mec* determinant comprises an unusual cluster of direct repeats and codes for a gene product similar to the *Escherichia coli sn*-diester phosphodiesterase. J. Bact. **173**: 7416-7422.

16. **Saiki, R.K., S. Scharf, F. Faloona, K.G. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim** (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**: 1350-1354.

17. **Sambrook, J., F. Fritsch and T.E. Maniatis** (1989) Molecular cloning: a laboratory manual. 2nd Edition. Cold Spring Harbour Laboratory, Cold Spring Harbor, N.Y.

18. **Sompolensky, P.Z., Z. Samra, W.W. Karakawa, W.F. Vaun, R. Schneerson, and Z. Malik** (1985) Encapsulation and capsular types in isolates of *S. aureus* from different sources and relationships to phage types. J. Clin. Microbiol **22**: 828-834.

19. **Ubukata, K., S. Nakagami, A. Nitta, A. Yamane, S. Kawakami, M. Sugiura, and M. Konno** (1992) Rapid detection of the *mecA* gene in methicillin-resistant staphylococci by enzymatic detection of polymerase chain reaction products. J. Clin. Microbiol. **30**: 1728-1733.

20. **Ünal, S., J. Hoskins, J.E. Flokowitsch, C.Y.E. Wu, D.A. Preston and P.L. Skatrud** (1992) Detection of methicillin-resistant staphylococci by using the polymerase chain reaction. J. Clin. Microbiol. **30**: 1685-1691.

21. **Van Belkum, A., J. de Jonckheere and W. Quint** (1992) Genotyping *Naegleria spp.* and *Naegleria fowleri* isolates by interrepeat polymerase chain reaction. J. Clin. Microbiol. **30**: 2595-2598.

22. **Versalovic, J., T. Koeuth and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl. Acids Res. **19**: 6823-6831.

23. **Welsh, J. and M. McClelland** (1991) Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. Nucl. Acids Res. **19**: 5275-5279.

24. **Williams, J.G.K., A.R. Kubelik K.J. Livak, J.A. Rafalski and S.V. Tingey.** (1991) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. **18**: 6531-6535.

Adapted from: Eur. J. Clin. Microbiol. Infect. Dis. 1994;13:420-424

CHAPTER XI

COMPARISON OF FOUR GENOTYPING ASSAYS FOR EPIDEMIOLOGICAL STUDY OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS

Alex van Belkum, Rene Bax[1] and Gilles Prevost[2]

[1]Diagnostic Center SSDZ, Dept. of Molecular Biology, P.O. Box 5010, 2600 GA Delft, The Netherlands. [2]Université Louis Pasteur, Lab. de Bacteriologie, 3, Rue Koeberlé, 67000 Strasbourg, France.

SUMMARY

Methicillin-resistant *Staphylococcus aureus* strains (n=26) were genetically differentiated by ribotyping, pulsed field gel electrophoresis (PFGE) of DNA macrorestriction fragments, random amplified polymorphic DNA (RAPD) analysis and interrepeat polymerase chain reaction (IR PCR) assays. The results show that the PCR mediated assays are as discriminatory as PFGE typing, whereas ribotyping is the least powerful genotypic procedure. It is suggested that due to the experimental ease with which PCR testing can be performed, PCR fingerprinting is the method of choice for establishing clonal relationships among *Staphylococcus aureus* isolates.

INTRODUCTION

Genetic analysis of microorganisms provides a means for the determination of relationships between bacterial isolates (1). In the case of *Staphylococcus aureus*, a frequently encountered nosocomial pathogen, this molecular methodology has been used intensely in order to investigate the spread or persistence of a given strain (2). In particular, the methicillin-resistant version of *Staphylococcus aureus* (MRSA) has been studied in detail (3). It has been suggested that MRSA represents a universal clone (4), although this is being questioned in a more recent report (5). From MRSA typing studies it is obvious that the technology used in order to determine clonality (or not) is of major importance for the epidemiological conclusions drawn. For this reason several comparisons between typing strategies for MRSA have been presented (e.g. 6, 7). In the present paper we evaluate the applicability of four genotypic characterisations for the determination of clonality in MRSA. For a French collection of 26 MRSA isolates, the genetic interrelationships were previously determined by ribotyping, random amplified polymorphic DNA (RAPD) analysis and pulsed field gel electrophoresis (PFGE) of DNA macrorestriction fragments. Here we add interrepeat polymerase chain reaction (IR PCR) assays and present all results in a comparative fashion.

MATERIALS AND METHODS

Twenty-six MRSA strains were included in this study. MRSA were identified as Gram-positive cocci that were facultative anaerobes producing free coagulase and acetoin,

but deficient for ß-galactosidase production. Resistance to methicillin was considered relevant when the inhibition zone for 5 μg oxacillin disks was less than 20 mm. Strains were stored in brain heart infusion medium containing 10% horse serum and 10% glycerol. The procedures and results of ribotyping, PFGE and RAPD analyses have been presented before (6, 7). Interrepeat PCR was performed as described before for a set of Dutch MRSA strains (8). For PCR, primers homologous to enterobacterial repetitive intergenic consensus primers ERIC1 and ERIC2 (9), primer BG2 and the MecA2 consensus primer were applied in four different assays. All amplicons were analysed by electrophoresis through 1-2% agarose gels cast in 40 mM Tris.acetate pH 7.5, 2 mM EDTA buffer containing ethidium bromide. Gels were photographed using Polaroid Polaplan films. On the basis of the different DNA banding patterns, the strains could be divided into various PCR fingerprint groups.

RESULTS AND DISCUSSION

An overview of the results of ribotyping, PFGE analysis and RAPD is given in Table 1. Figure 1 gives a representative example of the results obtained by IR PCR. For all strains, the various genetic scores are shown and the differentiating power of the procedures is described in the legend. Prior to IR PCR for all 26 strains, strains 1, 5 and 10, were characterised for assay reproducibility by testing of 20 colonies per strain. For all colonies belonging to one strain, all PCR fingerprints were unequivocally identical (results not shown). As such, IR PCR appears to be an experimentally reliable DNA typing assay. The results gathered by four assays, indexed by capital lettering and summarised in an overall type given a roman number are also included in Table 1. This latter table surveys all results obtained by the four different genotypic characterisation methods for 26 French MRSA isolates (6,7).

From Table 1 several conclusions can be drawn. Since the strains were selected on the basis of their differing PFGE patterns, it is obvious that this assay was the most discriminative. It should be emphasised, however, that using restriction enzymes other than Sma I for the same collection of strains, a lower degree of resolution may result. Ribotyping was clearly less discriminatory. Taken together with the fact that this procedure is also technically spoken the most demanding, it is clear that the other procedures (PFGE and both types of PCR) should be preferred over ribotyping. When PFGE and PCR are compared, apparently the use of a single PCR assays does not allow a similar degree of resolution. Whereas PFGE distinguishes all isolates, PCR identifies somewhere between 9 and 20 types per assay. It seems that the degree of variability that can be monitored is strongly dependent on the choice of primer or primer combination. Performing PCR with different primers allows grouping of isolates, irrespective of strain origin or typing based on other tests. This is once more underlined by the fact that several strains which are identical in one or more of the PCR tests used are not epidemiologically

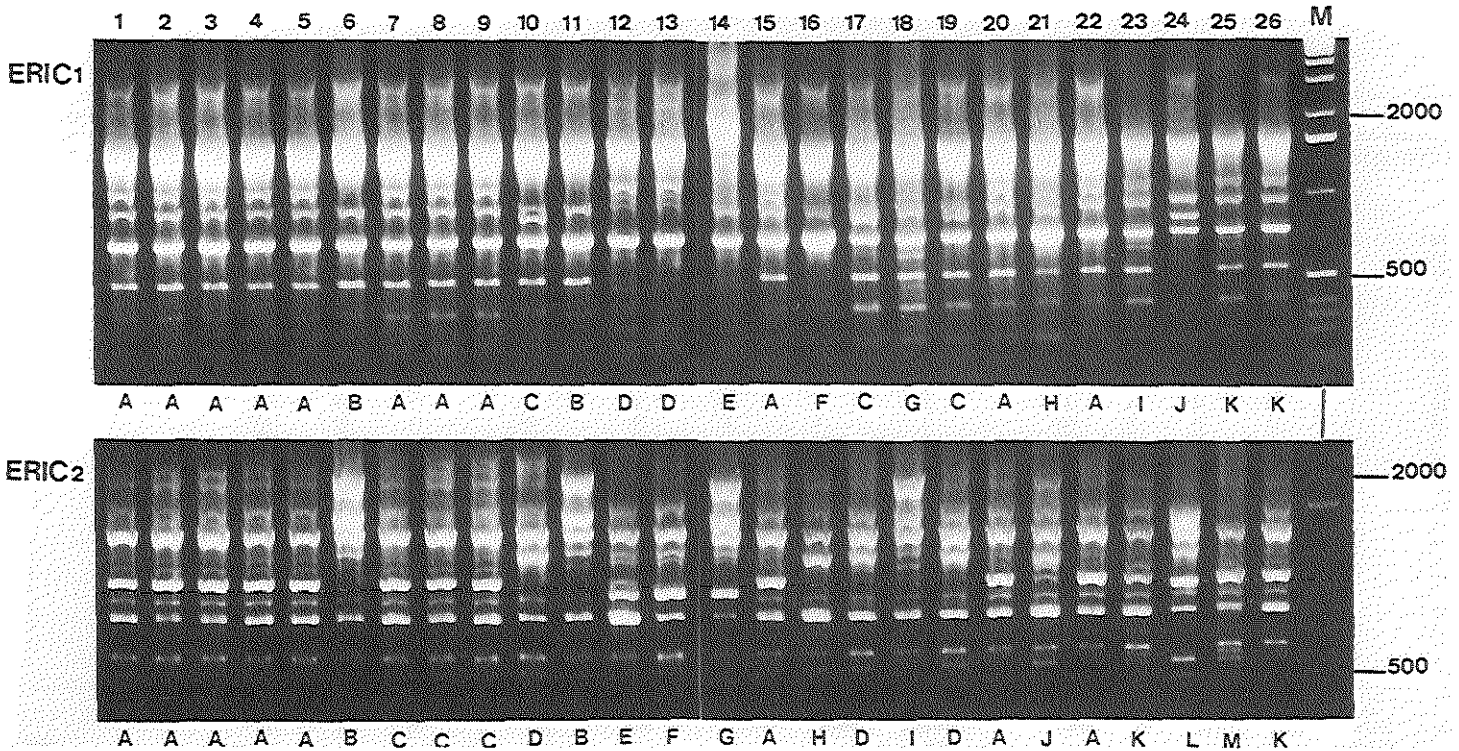


Figure 1 Example of the PCR fingerprints generated by the application of the repetitive motif primers ERIC1 (top panel) and ERIC2 (lower panel). The upper row gives sample numbering (1-26) whereas below the respective panels the codes, as also summarised in Table 1, are given. The lanes on the right contain molecular length markers, as is also indicated in basepairs.

Table 1 Genetic differentiation of 26 French MRSA strains by four genetic analysis procedures

strain code	PFGE	ribotype			RAPD		IR PCR								
		type Sma I	Eco RI	Cla I	ribo-Hind III	type overall	primer 2	primer 3	RAPD primer 5	overall	E1	E2	E1E2	IR PCR mecA2 BG2	overall
1		I	E1	C1	H1	I	A	A	A	I	A	A	A	A	I
2		II	E2	C1	H1	II	B	B	A	II	A	A	A	A	I
3		III	E1	C1	H1	I	B	C	A	III	A	A	B	B	II
4		IV	E1	C1	H1	I	B	D	B	IV	A	A	A	C	III
5		V	E1	C1	H1	I	C	E	A	V	A	A	A	A	I
6		VI	E5	C4	H4	III	D	F	C	VI	B	B	C	D	IV
7		VII	E2	C2	H2	IV	C	C	C	VII	A	C	A	C	V
8		VIII	E1	C1	H1	I	B	D	D	VIII	A	C	A	E	VI
9		IX	E1	C1	H1	I	B	D	D	VIII	A	C	A	C	V
10		X	E1	C1	H1	I	E	G	E	IX	C	D	D	E	VII
11		XI	E2	C2	H2	IV	F	H	F	X	B	B	E	D	VIII
12		XII	E1	C1	H1	I	G	I	D	XI	D	E	F	F	IX
13		XIII	E4	C4	H4	V	H	J	G	XII	D	F	F	-	X
14		XIV	E4	C1	H1	VI	I	K	H	XIII	E	G	G	F	XI
15		XV	E6	C1	H1	VII	J	L	I	XIV	A	A	A	C	III
16		XVI	E2	C2	H2	IV	K	M	J	XV	F	H	H	A	XII
17		XVII	E1	C1	H1	I	L	N	K	XVI	C	D	I	C	XIII
18		XVIII	E1	C1	H1	I	M	O	C	XVII	G	I	J	-	XIV
19		XIX	E3	C3	H3	VIII	N	N	L	XIII	C	D	D	G	XV
20		XX	E1	C1	H1	I	B	P	C	XIX	A	A	A	A	I
21		XXI	E1	C1	H1	I	O	Q	J	XX	H	J	K	H	XVI
22		XXII	E1	C1	H1	I	G	R	F	XXI	A	A	A	A	I
23		XXIII	E2	C3	H3	IX	B	E	M	XXII	I	K	L	-	XVII
24		XXIV	E1	C1	H1	I	P	S	N	XXIII	J	L	H	I	XVIII
25		XXV	E1	C1	H1	I	N	T	O	XXIV	K	M	L	-	XIX
26		XXVI	E3	C2	H2	X	Q	-	J	XXV	K	K	A	A	XX
nr of types:	26	6	4	4	10	17	20	15	25	11	13	12	9	20	

Note: The strain numbers correspond to those published previously (6, 7) and isolates have been selected on the basis of differing PFGE DNA fingerprints (column I, type I to XXVI). Ribotyping for these strains has been performed using three different restriction enzymes, whereas the two PCR tests have been performed using three (RAPD) and four (IR PCR) assays, respectively. In case of the IR PCR assays E1, E2, mecA2 and BG2 identify the ERIC1 primer, ERIC2 primer, methicillin resistance complex specific primer A2 and the arbitrary primer BG2, respectively. Overall scores (indicated below) give the combined discrimination of the respective assays. A small horizontal line implies that no data are available.

linked (results not shown). It appears that in methicillin resistant *Staphylococcus aureus* different genotypes (with geographically different frequencies of incidence) seem to co-exist. The difference between RAPD and IR PCR lies in the nature of the primers. Whereas RAPD analysis used arbitrary DNA oligonucleotides averaging 6-10 nucleotides in length, IR PR employs longer oligonucleotides, the sequence of which is usually based on repetitive sequence elements in prokaryotic genomes. After combination of PCR fingerprints, irrespective of the fact that in this study RAPD and IR PCR individually display a resolution lower than that of PFGE, all strains can be discriminated. By adding the RAPD overall score with the $MecA_2/BG_2$ PCR, even strains 8 and 9 can be discriminated. The discriminatory power of PCR can be modulated by altering primer choices and annealing temperatures. Since PCR is also technically less demanding than PFGE and will become common in medical microbiological diagnostics, PCR fingerprinting is the MRSA typing procedure of choice, especially when optimal primers (or combinations thereof) have been determined in preliminary experimental evaluations. The possibility to directly include whole bacterial cells in PCR amplification tests (10) will even enlarge the importance of PCR fingerprinting in microbial ecology and epidemiology.

REFERENCES

1. **Falkiner, F.R** (1988) Epidemiological typing: a user's view. J. Hosp. Infect. **11**: 303-309.
2. **Goh, S.H., A.S.K. Byrne, J.L. Zhang, and A.W. Chow** (1992) Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphisms. J. Clin. Microbiol. **30**: 1642-1645.
3. **Hackbarth, J.C., and H.F. Chambers** (1989) Methicillin-resistant Staphylococci genetics and mechanism of resistance. Antimicrob. Agents Chemother. **33**: 991-994.
4. **Kreiswirth, B., J. Kornblum, R.D. Arbeit, W. Eisner, J.M. Maslow, A. McGeer, D.E. Low, and R.P. Novick** (1993) Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. Science **259**: 227-230.
5. **Lencastre, H. de, A.M.S. Figueiredo, and A. Tomasz** (1993) Genetic control of population structure in heterogeneous strains of methicillin resistant *Staphylococcus aureus*. Eur. J. Clin. Microbiol. Infect. Dis. **12**: 13-18.
6. **Prévost, G., B. Jaulhac, and Y. Piemont** (1992) DNA fingerprinting by pulsed field gel electrophoresis is more effective than ribotyping in distinguishing among methicillin resistant *Staphylococcus aureus* isolates. J. Clin. Microbiol. **30**: 967-973.
7. **Saulnier, P., C. Bourneux, G. Prévost, and A. Andremont** (1993) Random amplified polymorphic DNA assay is less discriminant than pulsed field gel electrophoresis for typing strains of methicillin resistant *Staphylococcus aureus*. J. Clin. Microbiol. **31**: 982-985.
8. **Van Belkum, A., R. Bax, P. Peerbooms, W.H.F. Goessens, N. Van Leeuwen, and W.G.V. Quint** (1993) Comparison of phage typing and DNA fingerprinting by polymerase chain reaction for discrimination of methicillin-resistant *Staphylococcus aureus* strains. J. Clin. Microbiol. **31**: 798-803.

9. **Versalovic, J., T. Koeuth, and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19**: 6832-6831.
10. **Woods, C.R., J. Versalovic, T. Koeuth, and J.R. Lupski** (1993) Whole cell repetitive element sequence-based polymerase chain reaction allows rapid assessment of clonal relationships of bacterial isolates. J. Clin. Microbiol. **31**: 1927-1931.

Adapted from J. Clin. Microbiol 1995;33:1537-1547

CHAPTER XII

MULTICENTER EVALUATION OF ARBITRARY PRIMED PCR FOR TYPING OF *STAPHYLOCOCCUS AUREUS* STRAINS

Alex van Belkum, Jan Kluytmans, Willem van Leeuwen, Rene Bax[1], Wim Quint[1], Edith Peters[2], Ad Fluit[2,8], Christina Vandenbroucke-Grauls[2], Adriaan van den Brule[3], Hans Koeleman[3], Willem Melchers[4], Jacques Meis[4], Abdeslam Elaichouni[5], Mario Vaneechoutte[5], Francoise Moonens[6], Nicole Maes[6], Marc Struelens[6], Fred Tenover[7] and Henri Verbrugh

[1]Diagnostic Center SSDZ, Department of Molecular Biology, P.O. Box 5010, 2600 GA Delft, the Netherlands. [2]University Hospital Utrecht, Eijkman-Winkler Institute for Medical and Clinical Microbiology, P.O. Box 85.500, 3508 GA Utrecht, the Netherlands. [3]Free University Hospital,Department of Medical Microbiology, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands. [4]University Hospital Nijmegen, Department of Medical Microbiology, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands. [5]University Hospital, Department of Clinical Biology, De Pintelaan 185, B-9000 Gent, Belgium. [6]Hopital Erasme, Unite d'Epidemiologie, 808, Route de Lennik, 1070 Brussels, Belgium. [7]Centers for Disease Control and Prevention, Nosocomial Pathogens Laboratory Branch, 1600 Clifton Road, Atlanta Georgia 30333, USA. [8]U-Gene Research BV, Bolognalaan 40, 3584 CJ Utrecht, the Netherlands.

SUMMARY

Fifty-nine isolates of *Staphylococcus aureus* and a single strain of *Staphylococcus intermedius* were typed by arbitrary primed (AP) PCR. To study reproducibility and discriminatory abilities, AP PCR was carried out in seven laboratories with a standardised amplification protocol, template DNA isolated in a single institution, and a common set of three primers with different resolving power. The 60 strains could be divided into sixteen to thirty different genetic types, depending on the laboratory. This difference in resolution was due to differences in technical procedures (as shown by the deliberate introduction of experimental variables) and/or the interpretation of the DNA fingerprints. However, this did not hamper the epidemiologically correct clustering of related strains. The average number of different genotypes identified exceeded those of the more traditional typing strategies (Tenover et al. 1994. Comparison of traditional and molecular methods of typing of *Staphylococcus aureus*. J. Clin. Microbiol. 32:407-415). Comparison of AP PCR with pulsed-field gel electrophoresis (PFGE) indicated the existence of strains of constant PFGE but variable AP PCR types. The reverse (constant AP PCR and variable PFGE patterns) was also observed. This indicates additional resolution for combined analyses. It is concluded that AP PCR is well suited for genetic analysis and monitoring of nosocomial spreading of staphylococci. The interlaboratory reproducibility of DNA banding patterns and intralaboratory standardisation need improvement.

INTRODUCTION

Numerous procedures for comparison of bacterial isolates have been developed (for reviews, references 2, 11, 13 and 15). These procedures are important in investigations of strain origin, clonal relatedness among strains, and epidemiology. For *Staphylococcus aureus*, it has been demonstrated that most of the typing procedures can be applied successfully to obtain epidemiologically useful data. Tenover et al. (22) compared twelve typing strategies and concluded that DNA-based typing methods and immunoblotting are best suited for epidemiological analyses. With the exception of biotyping, which appeared to produce too many subtypes, no single technique proved overtly superior or inferior. When all procedures were compared, unrelated strains were grouped with differing frequencies. This comparative analysis of typing procedures provides a reference scheme for rating novel typing strategies against the more established methods.

Recently, a large number of reports describing the use of the polymerase chain reaction (PCR) for genetic typing of medically important microorganisms (for surveys, references 23 and 24) have appeared. By arbitrarily amplifying variable regions in the bacterial genome (arbitrarily primed (AP) PCR), isolate-specific DNA fingerprints can be obtained in a rapid and reproducible manner. In most cases, these analyses are not accompanied by detailed comparisons with the results of alternative typing procedures. For *S. aureus*, for instance, several studies have compared AP PCR with only a single other technique (20, 21, 25, 26).

The present study was undertaken to determine the reproducibility and discrimiatory abilities of AP PCR when compared to other staphylococcal typing procedures. To this aim, the *S. aureus* strains that were studied previously by Tenover et al. (22), were typed in multiple AP PCR assays with three different arbitrary primers, guided by a standard amplification protocol and performed independently in seven different laboratories.

MATERIALS AND METHODS

Bacterial strains and description of isolates: Fifty-nine isolates of *S. aureus* were included in this study. All isolates were identified and confirmed to be *S. aureus* by standard biochemical methods (12). The strains have been described before (22), and forty of them were derived from five well- documented outbreaks. The nineteen additional isolates are epidemiologically non-linked. A single isolate of *S. intermedius* was included.

The sixty strains were divided into three groups (SA, SB and SC) some of whose key features will be summarised below (Table 1). Group SA contains the strains involved in an outbreak, occurring in two nursing homes (strains labelled NH1 or NH2). Strain SA-04 is ATCC 12600 (American Type Culture Collection, Rockville, Md.). This set also contains a number of independent isolates from the Centers for Disease Control and Prevention, the *S. intermedius* strain, and three isolates of phage type 47/54/75/77/83A. These latter strains were isolated in three different American states during three different

years.

Group SB contains strains from outbreaks I and II, eight unrelated strains, and, again, *S. aureus* ATCC 12600 (SB-07). Strains from outbreak I are methicillin resistant and were isolated in the Iowa Veterans Affairs Medical Center (18). Outbreak II was related to a contaminated anaesthetic (6).

Group SC contains strains from outbreaks III and IV, an unrelated control, and ATCC 12600 (SC-03). Outbreak IV was again anaesthetic related (6), although it differed from outbreak II. Outbreak III was caused by ten methicillin-resistant strains obtained from the Sepulveda Veterans Affairs Medical Center, Sepulveda, California (9).

Bacterial typing studies: All isolates were typed previously by a number of procedures (22). Antibiograms and biotypes were determined, and bacteriophage sensitivity was assayed. Restriction fragment length polymorphisms (RFLP) were screened using enzymatic digestion of plasmid DNA, variable gene probes, or DNA probes derived from insertion elements (IS mapping). DNA macrorestriction fragments were separated by field inversion gel electrophoresis (FIGE) and pulsed-field gel electrophoresis (PFGE). Multilocus enzyme electrophoresis (MLEE) and immunoblotting were also performed, as were ribotyping and restriction enzyme analysis of PCR fragments derived from the staphylococcal coagulase gene.

PCR Multicenter study design: Participants were from seven institutions: two Belgian institutes (Hopital Erasme, Brussels; University Hospital of Ghent, Ghent), and five Dutch hospitals (University Hospital Radboud, Nijmegen; University Hospital Utrecht, Utrecht; Diagnostic Centre SSDZ, Delft; Free University Hospital, Amsterdam; University Hospital Dijkzigt, Rotterdam). The study was coordinated at the Dijkzigt Hospital, where the AP PCR assays were performed in duplicate by two individuals following slightly different experimental protocols. All participants had experience in performing AP PCR. This guarantees intralaboratory reproducibility of the assays. For this reason, the participants were also allowed to process the *S. aureus* DNA samples

---

Note to Table 1: Columns Ia through VII give the AP PCR data as determined in the different institutions. Data given in a certain column may be different from the same character in another column. Out, outbreak; NO, not in related cluster; I to IV, outbreak number; NH1/2, nursing home pseudo outbreak; Ox, oxacillin susceptibility; An bi, antibiogram; S, susceptible; R, resistant; INT, *S. intermedius* biotype; Pla mid, plasmid restriction profile; NP, no plasmids; Ri ty, ribotyping with *HindII* and *ClaI*; IS, insertion sequence; NH, no hybridisation; RFLP, restriction fragment length polymorphism; Coag PCR, coagulase gene PCR; PFGE, pulsed field gel electrophoresis; FIGE, field inversion gel electrophoresis; Im, immunoblotting; MLEE, multilocus enzyme electrophoresis. The strain numbers that are underlined and bold denominate three isolates of a single ATCC strain. The results of the PCR mediated fingerprinting are given per institute, which are encoded Ia through VII. The three letter code summarises the data per primer (first digit, primer 1; second, primer 7; third, primer E_2).

TABLE 1 GENERAL SURVEY OF PHENOTYPIC AND GENOTYPIC TYPING DATA IN COMPARISON WITH THE RESULTS FROM THE MULTICp1202XSTUDY ON AP PCR MEDIATED DNA FINGERPRINTING FOR 60 *STAPHYLOCOCCUS* STRAINS

Str.	Out	O x	Phage type	An. bi.	Bio type	AP PCR datasets Ia	Ib	II	III	IV	V	VI	VII	Pla mid	Ri ty	PF GE	FI GE	Im	ML EE	IS	Coag PCR	RFLP type
SA-16	NO	S	NR	I	INT	HEH	FEF	HEG	GDH	FDF	FDG	GFG	DDC	NP	de	I	VII	K	F	NH	0.0	NH:NH:NH:NH
SA-04	NO	S	6/47/54/75	B	A2B	BBB	BBB	BBB	BBB	BBB	BBC	BCC	BBA2	B	fi	E	IV	D	E	NH	2.1	NH:X:4:NH
SA-12	NO	R	47/54/75/77/83A	G	A3B	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA1	NP	bb	J	IC2	A	A5	C	9.0	I:A:1:NH
SA-18	NO	R	47/54/75/77/83A	J	A3B	AAA	AAA	AAA	AAA	AAA	AAA	ABA	AAA1	I	bb	J	IC3	A2	A3	C	9.0	I:A:1:NH
SA-20	NO	R	47/54/75/77/83A	K	A3B	AAA	AAA	AAA	AAA	AAA	AAA	ABB	EAA1	J	bb	J	IC1	A1	A1	C	9.0	I:A:1:NH
SA-06	NO	I	NR	C	A3B	CAC	AAC	CAC	CAC	AAA	AAB	ABB	AAA1	C	aa	C	III	A4	A4	B	9.0	II:NH:1:a
SA-07	NO	S	53/+	D	H4	DAD	AAC	DAC	AAD	CAA	AAD	CBA	AAA1	D	bc	B	V	C	A2	NH	9.0	NH:NH:1:NH
SA-08	NO	R	54/75/77/81	E	I2B	ECE	CCD	ECD	DBE	DBC	CBE	DDD	CB'B	E	ed	G	IIA	E1	D1	D	7.0	I:NH:6:NH
SA-11	NO	R	NR	F	A2B	ECF	CCD	ECE	DBF	DBD	CBF	DDE	CB'B	E	gd	F	IIB	E2	D2	G	7.0	II:NH:6:NH
SA-01	NH1	R	54/77	A1	A1B	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA1	A	aa1	K1	IB	A1	A1	A	9.0	I:A:5:a
SA-09	NH1	R	54/77	A	A1B	AAA	AAA	AAA	AAA	AAA	AAD	ABA	AAA1	NP	aa1	K2	IB	A1	A1	A	9.0	I:A:5:a
SA-03	NH1	R	47/54/75/77	A2	A3B	AAA	AAA	AAA	AAA	AAA	AAB	ABB	AAA1	NP	aa	A	IA	A	A1	C	9.0	I:A:1:NH
SA-13	NH1	R	54/77	A3	A1B	AAG	AAA	AAA	AAA	AAA	AAA	AAA	AAA1	G	aa	A	IA	A3	A2	A	9.0	I:A:1:a
SA-14	NH1	S	54/75/77	H	B1B	GDG	EDE	GDF	FCG	-CE	ECA	FEF	ACA3	H	ei	H	VI	E3	C	NH	9.0	NH:NH:1:NH
SA-19	NH1	R	54/77	A4	G1B	AAA	AAA	AAA	AAA	AAA	AAB	ABB	EAA1	A	aa1	K3	IB	A1	A1	A	9.0	I:A:1:a
SA-17	NH2	R	54/75/77	A	C3B	AAA	AAA	AAA	AAA	AAA	AAB	ABB	EAA1	A	aa	A	IA	A	A1	A	9.0	I:A:1:a
SA-02	NH2	R	75/77	A	A3B	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA1	A	aa	A	IA	A	A1	A1	9.0	I:A:1:b
SA-15	NH2	R	77	A	A3B	AAA	AAA	AAA	AAA	AAA	AAA	AAB	AAA1	A	aa	A	IA	A1	A5	A1	9.0	I:A:1:a
SA-05	NH2	R	77	A	A3B	AAA	AAA	AAA	AAA	AAA	AAB	ABB	AAA1	A	aa	A	IA	A	A1	A	9.0	I:A:1:a
SA-10	NH2	R	77	A	A3B	FAA	DAA	FAA	EAA	EAA	DAA	EAA	JAA1	A	aa	D	ID	A1	B	A	9.0	I:A:1:a
SB-07	NO	S	6/47/54/75	C	A2B	BBB	BBB	BBB	JBB	BBH	-BC	BCC	BBA1	D	ei	D	IIB3	D'	B3	NH	2.1	NH:X:4:NH
SB-03	I	R	75/+	A	C4	AAA	AAA	AFA	HEA	AE-	AEA	HGA	EEA1	C	aa	A	IA	A6	A1	E	9.0	I:A:1:a
SB-05	I	R	75/+	A	A4	AAA	AAA	AFA	HEA	AEA	AEA	HGA	EEA1	C	aa	A	IA	A6	A1	E	9.0	I:A:1:a
SB-10	I	R	75/+	A	A4	AAA	AAA	AFA	HEA	AEA	AEK	HGA	HEA1	C	aa	A	IA	A6	A1	E	9.0	I:A:1:a
SB-12	I	R	75/+	A	C4	AAA	AAA	AFA	HEA	AEL	AEK	HGA	AAA1	C	aa	A1	IA	A6	A1	E	9.0	I:A:1:a
SB-15	I	R	75/77/83	A	C4	AAA	AAA	AFA	HEA	AEL	AEA	HGA	AAA1	C	aa	A	IA	A6	A1	E	9.0	I:A:1:a
SB-19	I	R	75/+	A	A4	AAA	AAA	AFA	HEA	AEL	AEA	HGA	AAA1	C	aa	A	IA	A5	A1	E	9.0	I:A:1:a
SB-20	I	R	75/+	A	A4	AAA	AAA	AFA	HEA	AEL	AEK	HGA	AAA1	C	aa	A		A5	A1	E	9.0	I:A:1:a
SB-01	NO	R	75/77	A	A4	AAA	AAA	AFA	HEA	AEA	AEA	HGA	EEA1	A	aa	A1	IB1	A5	A1	E	9.0	I:Y:1:a

SB-16	NO	R	75/77/83A	A	A4	AAA	AAA	AFA	HEA	AEL	AEA	HGA	AAA1	A	aa	A1	IB1	A5	A1	E	9.0	I:Y:1:a
SB-18	NO	R	75/+	A	C4	AAA	AAA	AFA	HEA	AEL	AEA	HGA	AAA1	J	aa	A	IA	A7	A1	E1	9.0	I:A:1:a
SB-17	NO	I	96	E	B3B	LDL	JIM	NJM	OCQ	LCO	KCM	NKM	ACA6	I	fj	E	IV	G	A2	NH	6.0	NH:NH:1:NH
SB-14	NO	R	47/54/75/77/83A	A1	A3B	AAG	AHL	AAL	HAP	AAN	AAA	AAF	AAA3	H	ea	A2	IB2	A5	A3	D	9.0	I:A:1:NH
SB-08	NO	S	95	B1	C4	JGJ	HBI	JHI	KGM	IGI	IGI	JII	GFG	E	dd1	F	III	E5	C	NH	2.0	NH:NH:1:NH
SB-02	II	S	3A/55	B	B1B	IFI	GFH	IGH	IFK	GFG	HFH	IHH	FBD	B	bb	B	IIA	D1	B1	NH	6.0	NH:NH:7:NH
SB-04	II	S	3A/55	B	D1B	IFI	GFH	IGH	IFL	HFG	-FH	IHH	FBD	B	bb	B	IIA	D1	B1	NH	6.0	NH:NH:7:NH
SB-06	II	S	3A/55	B	B1B	IFI	GFH	IGH	IFK	HFG	HFH	IHH	FBD	B	bb	B	IIA	D1	B1	NH	6.0	NH:NH:7:NH
SB-11	II	S	3A/55	B	B3B	FFK	GFK	LGK	MFO	KHK	JFL	LHK	ABE	G	b1b	C	IIB2	D2	B1	NH	14.0	NH:NH:7:NH
SB-09	NO	S	3A	D	D3B	BFI	IDJ	KIJ	LBN	JBJ	BBJ	KJJ	HBE	F	bb	B	IIA	D1	B1	NH	6.0	NH:Z:7:NH
SB-13	NO	S	3A	B2	D3B	KFI	GGJ	MIJ	NBN	RBM	BBJ	MIL	ABF	G	bb	B1	IIB1	E6	B2	NH	6.0	NH:NH:7:NH
SC-03	NO	S	6/47/54/75	C	A2B	BBB	BJB	BBB	JBB	OBP	LBC	BCP	ABA4	C	ai	C	III	D	B	NH	2.1	NH:NH:4:NH
SC-01	III	R	75	A	A1B	MFB	BJB	OBB	PBB	MBP	LBB	OLN	ABA4	A	ab	A	IA	F	A1	F	10.0	I:A:4:a
SC-04	III	R	75	A	A1B	MFB	BJB	OBB	PBB	MBP	LBB	OLN	ABA4	D	ab	A	IA	F	A1	F	10.0	I:A:4:a
SC-05	III	R	NR	A1	A1B	MFB	BJB	OBB	PBB	-BP	LBB	OLN	ABA4	D	ab	A	IA	F	A1	F	10.0	I:A:4:a
SC-09	III	R	75	A	A1B	MFB	BJB	OBB	PBB	MBP	LBN	OCN	IBA5	D	ab	A	IA	F	A1	F	10.0	I:A:4:a
SC-11	III	R	75	E	A1B	MFB	BJB	OBB	PBB	MBP	LBN	OCN	IBA5	NP	ab	A	IB	F	A1	NH	10.0	I:A:4:NH
SC-12	III	R	75	A2	A1B	MFB	BJB	OBB	PBB	MBP	LBN	OLN	IBA5	A	ab	A	IA	F	A1	F	10.0	I:A:4:a
SC-14	III	R	75	A2	B2B	MFB	BJB	OBB	PBB	MBP	LBN	OLN	IBA5	A	ab	A	IA	F	A2	F	10.0	I:A:4:a
SC-15	III	R	75	A	A1B	MFB	BJB	OBB	P-B	MBP	LBN	OCN	IBA5	D	b2b	A	IA	F	A1	F	10.0	I:A:4:a
SC-17	III	R	75	A	A1B	MFB	BJB	OBB	P-B	MBP	LBN	JIN	IBA5	A	ab	A	IA	F	A1	F	10.0	I:A:4:a
SC-20	III	R	75	A	A1B	MFB	BJB	OBB	PB-	MBP	LBN	JIN	IBA5	D	ab	A	IA	F	A1	F	10.0	I:A:4:a
SC-08	NO	S	NR	B	B3A	JHD	HKI	JHI	KGM	PII	MGI	JIH	GFG	E	b1g	B1	II	E7	A3	NH	2.0	NH:NH:1:NH
SC-02	IV	S	52/52A/80/47/54 83A/84/95	B	E1B	NGJ	HKI	JHI	KGM	NII	MGI	JIO	GFG	B	bg	B	II	E7	C1	NH	2.0	NH:NH:1:NH
SC-06	IV	S	95	B	J1B	JHD	HKI	JHI	KGM	PJI	MGI	JIH	GFG	B	bg	B	II	E7	C1	NH	2.0	NH:NH:1:NH
SC-07	IV	S	95	D	I1A	JHD	HKI	JHI	KGM	PJI	MGI	JIQ	GFG		bg	B	II	E7	C1	NH	2.0	NH:NH:1:NH
SC-10	IV	S	52A/79/80/47/54 75/77/83A/95	B	I2A	JHD	HKI	JHI	KGM	-II	MGI	JIH	GFG	B	bg	B	II	E7	C1	NH	2.0	NH:NH:1:NH
SC-13	IV	S	95	B1	I1B	JHD	HKI	JHI	KGM	NII	MGI	JII	GFG	B	bg	B	II	E7	C1	NH	2.0	NH:NH:1:NH
SC-16	IV	S	95	B1	I1B	JHD	HKI	JHI	KGM	PJI	MGI	OLI	GFG	B	ag	B	II	H	D1	NH	2.0	NH:NH:1:NH
SC-18	IV	S	95	F	I3B	JHD	HKI	JHI	KGM	PJI	MGI	OLI	GFG	B	bg	B	II	E7	C1	NH	2.0	NH:NH:1:NH
SC-19	IV	S	95	B1	D1A	JHD	HKI	JHI	KGM	PII	MGI	JOI	GFG	B	bg	B	II	E7	D2	NH	2.0	NH:NH:1:NH
NUMBER OF TYPES	3	19		11	15	20	16	18	21	30	21	28	19	10	15	11	7	7	6	8	8	17

according to their own, optimised AP PCR protocol. Results are presented anonymously, datasets are numbered from I through VII (sets Ia and Ib derive from the coordinating laboratory).

To prevent interlaboratory variation due to different DNA extraction protocols, bacterial DNA, and not the organisms, was distributed from the coordinating center to the participating laboratories. Primers were aliquoted in Rotterdam as well and shipped together with the DNA preparations. In this way, the variables of bacterial cultivation, DNA isolation, and primer quality were controlled. This implies, however, that the results obtained during this study may differ from those that would have been obtained if bacterial strains, rather than DNA, had been distributed. DNA amplification was performed in the different laboratories with the locally available equipment and PCR ingredients. Gels containing the amplified DNA were photographed, and the results were interpreted locally according to the researchers' individual standards. Generally, differences in the number of bands indicated a novel type. Variations in band-staining intensities were disregarded. Interpretation was performed without knowledge of epidemiological relatedness. The fingerprint types were transformed in a cumulative three-letter code (one letter per type per primer) and sent to Rotterdam, where a comparative analysis was carried out. Results were studied with respect to reproducibility of the DNA fingerprints (and the accompanying interpretation and strain grouping), relation to the results obtained by other typing procedures, and epidemiological value. When possible, data were further analyzed with Gelcompar Software (Applied Maths, Kortrijk, Belgium). Pictures were digitised with a Hewlett-Packard Scanjet IIc document scanner. After conversion and visual normalisation, the data were analyzed. Degrees of homology were determined by Dice comparisons, and clustering correlation coefficients were calculated by the unweighted pair group method with arithmetic averages (UPGMA).

Arbitrary primed PCR: A description of the three PCR-related procedures will be given below. This protocol served as a reference manual. Specific deviations of this protocol are summarised per institute in Table 2.

Cultivation of bacteria and isolation of DNA: Bacteria were grown in suspension in Brain Heart Infusion (BHI) broth for 18 h at 37°C. Approximately 100 ul of a bacterial pellet was suspended in 150 ul of 25 mM Tris.HCl pH 8.0, 50 mM glucose, 10 mM EDTA. Lysostaphin (75 ul of a 100 ug/ml solution) was added, centrifuged and the mixture was incubated at 37°C for 1 h. Spheroplasts were lysed by the addition of 1 ml 4 M guanidinium isothiocyanate, 50 mM Tris.HCl pH 6.4, 3 mM EDTA, 1% Triton-X100. To immobilize and purify the DNA, 50 ul of a Celite suspension (0.2 g/ml, Janssen Pharmaceuticals, Beerse, Belgium) was added. The entire mixture was shaken for 15 seconds and incubated at room temperature for 10 min. After centrifugation, the supernatant was discarded and the pellet was washed. The Celite pellet was dried in vacuo. Between 100 and 400 ul of 10 mM Tris.HCl pH 8.0, 1 mM EDTA was added, and DNA was eluted

Table 2 Survey of experimental variables with respect to PCR fingerprinting performedin the different participating laboratories

	Ia	Ib	II	III	IV	V	VI	VII
Inc. volume	100	100	100	50	100	100	50	50
Polymerase	Tth	Tth	Tth	Tth	Taq	Thp	Taq	Taq
Unit polymerase	0.20	0.20	0.25	0.10	1.25	0.25	1.00	0.25
Mg^{++}conc (mM)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
dNTP conc (uM)	40	40	40	100	200	40	200	200
KCl conc (mM)	50	50	50	50	50	*	50	50
PCR machine	Biomed	Biomed	Biomed	Hybaid	Biomed	P.E.	P.E.	P.E.
Sample size	30%	30%	30%	50%	25%	30%	30%	30%
Polaroid	57/3000	57/3000	52/400	665/80	665/80	667/3000	667/3000	665/80
Type agarose	Pron.	Pron.	Pron.	MP	Pron.	MP	UP	MP
Perc. agarose	1.5%	1.5%	1.5%	1.5%	1.5%	1.5%	1.5%	1.5%
Voltage	150	150	200	100	100	100	130	150
Migration	17 cm	10 cm	10 cm	8 cm	10 cm	10 cm	8-9 cm	8-10 cm
+ eth. bromide	-	-	+	+	+	+	-	+

Note: The description of the photographs describe the type (upper line) and the sensitivity in ASA values. The sample volume indicates the amount of the amplification reaction that has been separated electrophoretically. KCl and Mg^{++} concentrations are in millimolar, the dNTP concentration is given in micromolar. The polymerase brands are described (Thp: Thermoperfect DNA polymerase, Integro, Zaandam, the Netherlands; Tth: Supertaq, Sphaero Q, Leiden, The Netherlands; Taq: Taq polymerase, Cetus, USA) and the amount per reaction is given in units. The incubation volume is in microliters. *: in this case no KCl was present, instead 50 mM $(NH_4)_2SO_4$ was included. Pron: Pronarose, Hispanagar, Burgos, Spain; MP multi purpose agarose Boehringer Mannheim; UP: Ultra Pure Agarose, Gibco/BRL, Breda, the Netherlands. The presence (+) or absence (-) of ethidium bromide during the electrophoresis is indicated.

by incubation at 56°C for 10 min, interrupted by short periods of vortexing. The supernatant containing the DNA was separated from the Celite by centrifugation. The DNA concentration was determined by spectrophotometry at 260 nm and DNA was stored at -20°C). Stock solutions of bacterial DNA were adjusted to a concentration of 5 ng/ul.

Polymerase Chain Reaction: When Tth polymerase (SuperTaq, HT Biotechnology, Cambridge, United Kingdom) was used, the amplification conditions described below guaranteed optimal performance for this particular enzyme. The use of other enzymes usually required modification of the buffer conditions used during PCR (Table 2) and may lead to different AP PCR results. PCR was performed in a buffer system containing 10 mM Tris.HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100, 0.2 mM deoxynucleotide triphosphates, 50 pmoles of primer and 0.2 units of the Tth polymerase, to which DNA is added (50 ng per amplification). The PCR mixtures were overlaid with 100 ul of mineral oil. Cycling was performed in Biomed PCR machines (Model 60) and consisted of the following steps: predenaturation at 94°C for 4 min followed by 35 cycles of 1 min 94°C, 1 min 25°C, 2 min 74°C. Amplified DNA was stored at -20°C. The primers used to discriminate *S. aureus* strains were RAPD1 (GGTT-GGGTGAGAATTGCACG), RAPD7 (GTGGATGCGA) and ERIC2 (AAGTAAGT-

GACTGGGGTGAGCG) (25, 26, 28).

Electrophoresis: Amplification products were separated by electrophoresis in 1.5% agarose gels (5 mm thick; Hispanagar, Sphaero Q, Leiden, the Netherlands). Gels were run in 0.5 x tris-borate-EDTA (TBE) at a constant current of 100 mA for two h. Prior to electrophoresis, samples were mixed with a fivefold concentrated layer mix consisting of 50% glycerol in water and 0.8 mg bromophenolblue per ml. Then thirty-five ul of the amplified material was loaded on the gel, and a molecular weight marker was run in parallel with the AP PCR samples. Gels were stained after electrophoresis by addition of 10 ul ethidiumbromide (10 mg/ml) to a total volume of 300 ml of 0.5xTBE. The gels were photographed with a Polaroid MP4 Landcamera and Polaroid 57 High Speed films, with an exposure time of 0.125 to 0.25 sec (diaphragm F5.6). Table 2 surveys the differences among the electrophoresis conditions as applied in the different laboratories.

RESULTS

PCR Fingerprinting: An overview of the typing results is given in Table 1. The AP PCR data are displayed in separate columns, one per participating research center, except for the coordinating laboratory, where the assays were performed in duplicate (Ia and Ib). Figure 1 gives an example of a complete set of gel pictures obtained for the 60 strains with the three AP PCR primers. Table 3 displays the number of genotypes that was detected in the participants' laboratories. When primer 1 was used, the overall number of types varied from 10 to 17, with a mean of 14 types. The mean numbers for primer 7 and E2 were 9 and 14, respectively. With this set of strains, the discriminatory power of primers 1 and E2 is over 60% higher than of primer 7. The overall number of DNA bands generated per primer does not correspond to the number of detectable genotypes. Dataset IV, displaying 30 different genocodes (Tables 1 and 3), was produced from relatively small numbers of DNA fragments synthesised: 7, 5, and 7 types for primers 1, 7, and E2, respectively. These are smaller numbers than those found by the group describing the smallest number of genotypes (dataset Ib, with 16 types deduced from fingerprints consisting of 9, 9 or 11 bands for individual fingerprints). The maximum number of bands is observed when primer 1 was used. The mean score for this primer, averaged among the groups, is 11.4 DNA fragments. For primers 7 and E2, these numbers are 6.6 and 9.6 fragments, respectively. There is no apparent variation in the average length of the fragments, as demonstrated by a survey of the cumulative results obtained by application of primer 1 (Figure 2). Although most of the types are found in all laboratories, some additional bands give rise to additional genotypes.

Discordant results can be observed. The data obtained with primer E2 seemed to be the most variable (results not shown). When the overall number of combined genocodes is considered, major differences among laboratories are encountered. The number of types varies between 16 and 30, with a mean of 22 types identified.

When the length of the DNA fragments generated by the individual PCR tests were

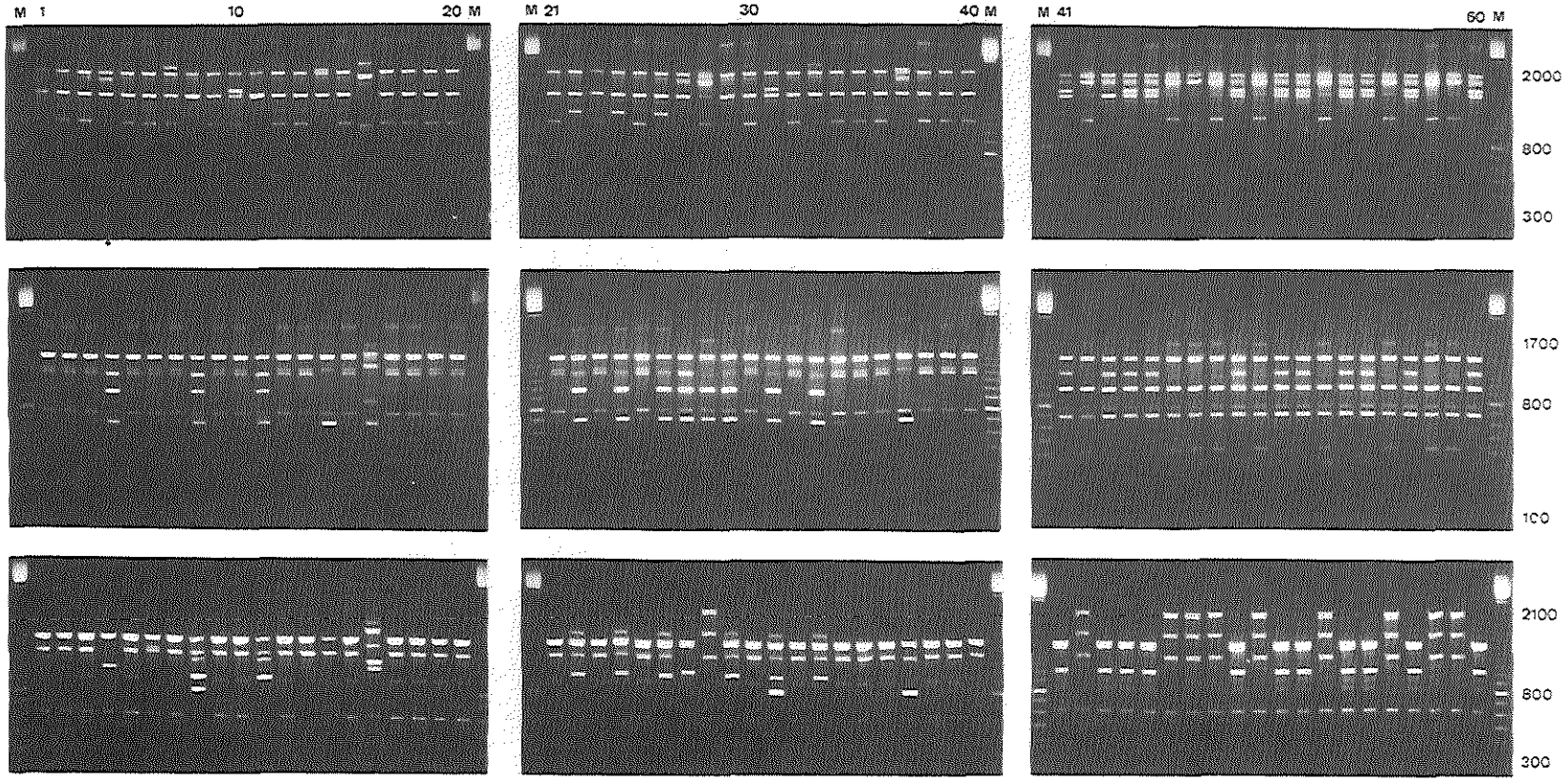


Figure 1 AP PCR for the 60 staphylococcal strains: survey of the experimental results belonging to dataset Ia. From left to right the strains are numbered according to Table 1, from top to bottom the primer species (primer 1, 7 and E2) has been varied. The outer lanes in all nine panels contain molecular length markers, the size of which is indicated on the right. The interpretation of the banding patterns is given in Table 1 in the column identified as Ia.

investigated, primer 1 was found to generate amplicons with an overall length of approximately 11,000 bp. For primers 7 and E2, these values are 4,000 and 5,500 bp, respectively. These differences are not reflected in the overall number of detectable PCR types (which is fourteen for both primer 1 and E2). Primer 7, detecting the smallest number of types, is also associated with the shortest cumulative length of the DNA fragments synthesised. There is an apparent variation in the number and size of fragments generated per primer species. For primer 1, this number varies from 6.4 to 11.4 on average. The numbers for primers 7 and E2 are 3.7 to 6.6 and 6.0 to 9.6, respectively. In general, fragments vary in length from 0.15 to over two kbp.

Epidemiological considerations: Analysis of outbreak IV illustrates that the data obtained by 5 out of 7 laboratories group these isolates into a homogeneous genotype that is not encountered in the rest of the collection, with the exception of a single strain (SC-08). These data are similar to those obtained by oxacillin susceptibility testing, plasmid typing, ribotyping, PFGE, FIGE, immunoblotting, IS mapping, PCR typing and RFLP mapping (22). Two of the seven laboratories detected 3 to 6 different types in this group of 8 bacterial isolates. The results collected for the strains from outbreak III are similar. In this case, 4 of 7 datasets demonstrate the homogeneity of this subgroup. Three participants identified two or three different types. Interestingly, in two of these laboratories, where the same subtypes are established, the differences were limited to data obtained by only one of the PCR primers. The PCR data are in agreement with those obtained by the other typing techniques. The four strains from outbreak II are split into 2 types: 3 are identical (5 of 7 laboratories) or similar (2 of 7 laboratories), whereas a single strain (SB-11) appears different. The other datasets confirm this observation. Results with strains from outbreak I and the nursing home (NH1 and NH2) conform those of the other typing procedures. Since nineteen non-outbreak-related strains are included in the collection, this implies that the resolution of PCR fingerprinting varies from approximately 50% to nearly 90%, since between 9 and 16 unique types were identified depending on the institute. It is assumed that all nineteen non-outbreak-related strains are indeed genetically independent.

Typeability and reproducibility: All strains were typeable by PCR. Four of seven labs obtained 100% typeability; negative results seen by other participants were due to technical inadequacy not related to bacterial genome structure. The overall mean level of typeability was 99.5%. This makes PCR-mediated typing preferable in principle over phage typing, plasmid typing, some of the RFLP approaches, and IS mapping, which all leave an appreciable percentage of strains untypeable.

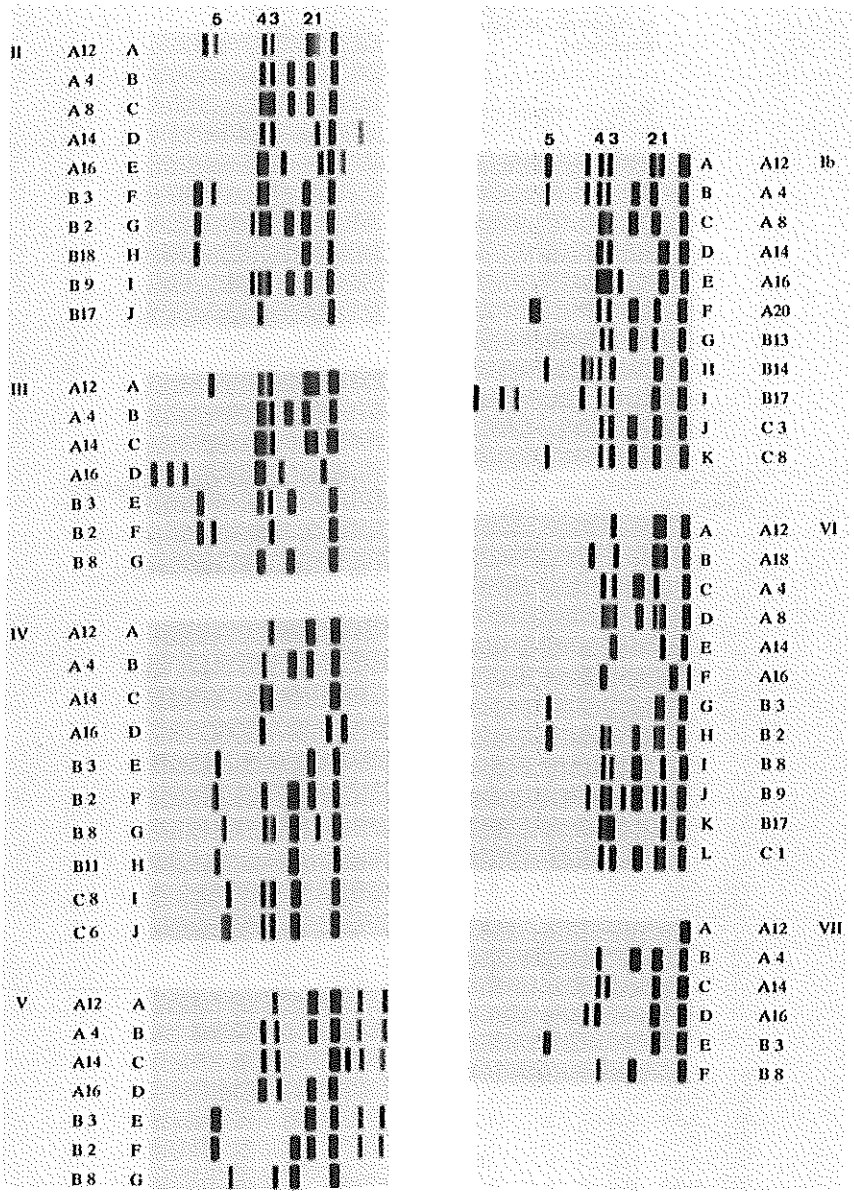


Figure 2 Survey of unique PCR fingerprints of staphylococcal DNA by primer 7. In panel Ib through VII a survey of the banding patterns as observed by the various participants is shown. Strain numbers and PCR genocodes are indicated alongside the panels as in Table 1. Note that the migration of DNA fragments displayed in panels Ib, VI and VII is differing from those in panels II to V. For comparison, some of the common bands are identified on top with a number (1 through 5). Dataset Ia has not been included in this comparison, in case of dataset VII pattern B' has been omitted.

Table 3 Number of genetic variants detected with the individual PCR primers or combinations thereof, as independently documented the 7 participating laboratories.

Partic. Center	primer 1	primer 7	primer E2	overall	unique strains
Ia	14	8	11	20	13
Ib	10	11	11	16	9
II	15	10	13	18	12
III	16	7	17	21	16
IV	17	10	16	30	15
V	13	7	14	21	11
VI	15	12	17	28	15
VII	10	7	12	19	11
average	14	9	14	22	13

Table 4 Comparison of PCR fingerprinting and PFGE on the basis of simplified genetic codes for the PCR assay.

St	Ib	IV	Ib	IV	PFGE	St	Ib	IV	Ib	IV	PFGE
A16	FEF	FDF	1	1	I	B18	AAA	AEK	3	6	A
A4	BBB	BBB	2	2	E	B17	JIM	LCO	6	7	E
A12	AAA	AAA	3	3	J	B14	AHL	AAN	7	3d	A2
A18	AAA	AAA	3	3	J	B8	HBI	IGI	8	8	F
A20	AAA	AAA	3	3	J	B2	GFH	GFG	9	9	B
A6	AAC	AAA	3a	3	C	B4	GFH	HFG	9	9a	B
A7	AAC	CAA	3a	3a	B	B6	GFH	HFG	9	9a	B
A8	CCD	DBC	4	4	G	B11	GFK	KHK	9a	10	C
A11	CCD	DBD	4	4a	F	B9	IDJ	JBJ	10	11	B
A1	AAA	AAA	3	3	K1	B13	GGJ	RBM	11	12	B1
A9	AAA	AAA	3	3	K2						
A3	AAA	AAA	3	3	A	C3	BJB	OBP	2a	13	C
A13	AAA	AAA	3	3	A	C1	BJB	MBP	2a	13a	A
A14	EDE	-CE	5	5	H	C4	BJB	MBP	2a	13a	A
A19	AAA	AAA	3	3	K3	C5	BJB	-BP	2a	13a	A
A17	AAA	AAA	3	3	A	C9	BJB	MBP	2a	13a	A
A2	AAA	AAA	3	3	A	C11	BJB	MBP	2a	13a	A
A15	AAA	AAA	3	3	A	C12	BJB	MBP	2a	13a	A
A5	AAA	AAA	3	3	A	C14	BJB	MBP	2a	13a	A
A10	DAA	EAA	3b	3b	D	C15	BJB	MBP	2a	13a	A
						C17	BJB	MBP	2a	13a	A
B7	BBB	BBH	2	2a	D	C20	BJB	MBP	2a	13a	A
B3	AAA	AE-	3	3c	A	C8	HKI	PII	2a	14	B1
B5	AAA	AEA	3	3c	A	C2	HKI	NII	8a	14a	B
B10	AAA	AEA	3	3c	A	C6	HKI	PJI	8a	15	B
B12	AAA	AEL	3	6	A1	C7	HKI	PJI	8a	15	B
B15	AAA	AEL	3	6	A	C10	HKI	-II	8a	14a	B
B19	AAA	AEL	3	6	A	C13	HKI	NII	8a	14a	B
B20	AAA	AEL	3	6	A	C16	HKI	PJI	8a	15	B
B1	AAA	AEA	3	3c	A1	C18	HKI	PJI	8a	15	B
B16	AAA	AEL	3	6	A1	C19	HKI	PII	8a	14	B

The data sets Ib and IV have been simplified by changing three- into one-letter codes. New types were defined when at least two characters from the three letter code had changed, single assay changes result in subtyping (a through d). Epidemiologically related clusters are indicated by returns, strain numbering is as in Table 1.

Discriminatory power: PCR fingerprinting was not able to detect differences between strain SA-12, SA-18, and SA-20. These strains are also identical by phage typing, ribotyping, PFGE, and IS mapping. Since these strains were derived from diverse origins, it seems likely that certain clones of *S. aureus* spread easily and remain genotypically constant. On the other hand, several other strains belonging to the same phagovar are differentiated by the PCR tests. As has been argued previously (26), PCR fingerprinting provides additional discrimination over that provided by phage typing.

The participants who detected the smallest number of types (n=16) clustered 37 out of 40 epidemiologically linked strains (including the NH1 and NH2 strains) correctly; however, 8 of the 20 unrelated isolates could not be distinguished from strains within the outbreak groups. When the dataset displaying the maximal number of PCR types (n=30) was evaluated, 29 of 40 strains were clustered. This is not an improvement when compared with the least discriminative data. Among the 20 unrelated strains, six genotypes were detected which were also found among the epidemiologically linked strains. Apparently, the rise in absolute number of detectable PCR genotypes adversely affects the correlation with the epidemiological data.

Comparison with pulsed field gel electrophoresis: PFGE is currently considered to be one of the most reliable and reproducible typing procedure allowing the detection of a high degree of DNA polymorphism (15). PCR and PFGE data were compared, the results are described in Table 4. First, the PCR codes for the two groups detecting the largest (dataset IV) and the smallest (dataset Ib) number of types were simplified. The three-letter code was condensed into a single digit, and new types were defined only when more than one individual AP PCR assay gave a different result. In case of a single change (from AAA to AAC, for instance) subtypes were defined. The results for set Ib were rearranged into 11 types and 5 subtypes, the data obtained for set IV defined 15 types and 9 subtypes. PFGE recognises 11 types (A through K) and 5 subtypes, equalling the numbers detected in the set Ib experiments. Nearly full epidemiological agreement exists between these latter data and PFGE results. The only difference occurs for the NH1 outbreak: PFGE found three instead of the expected two types (isolate 14 is also recognised as a deviant type by other procedures). The set IV data subdivide strains from outbreak I andIV and, as such, gave rise to an overestimation of the number of types.

Gelcompar analysis: Gelcompar analysis was disappointing. Of eight datasets, only four were accessible to scanning. Of these four datasets only one could be used for successful phylogenetic analysis. The other three composite pictures could not be analyzed because of lack of contrast, smiling of the gels, and low-resolution photography. In the single instance in which an phylogenetic tree could be constructed, it appeared that the result was in agreement with visual inspection and epidemiological data (Figure 3). The four sets of outbreak-related strains were clustered with homology percentages from 79 up to 93%, when data gathered with the three primers were combined. Clearly, Gelcompar analysis is heavily influenced by electrophoretic and photographic artifacts.

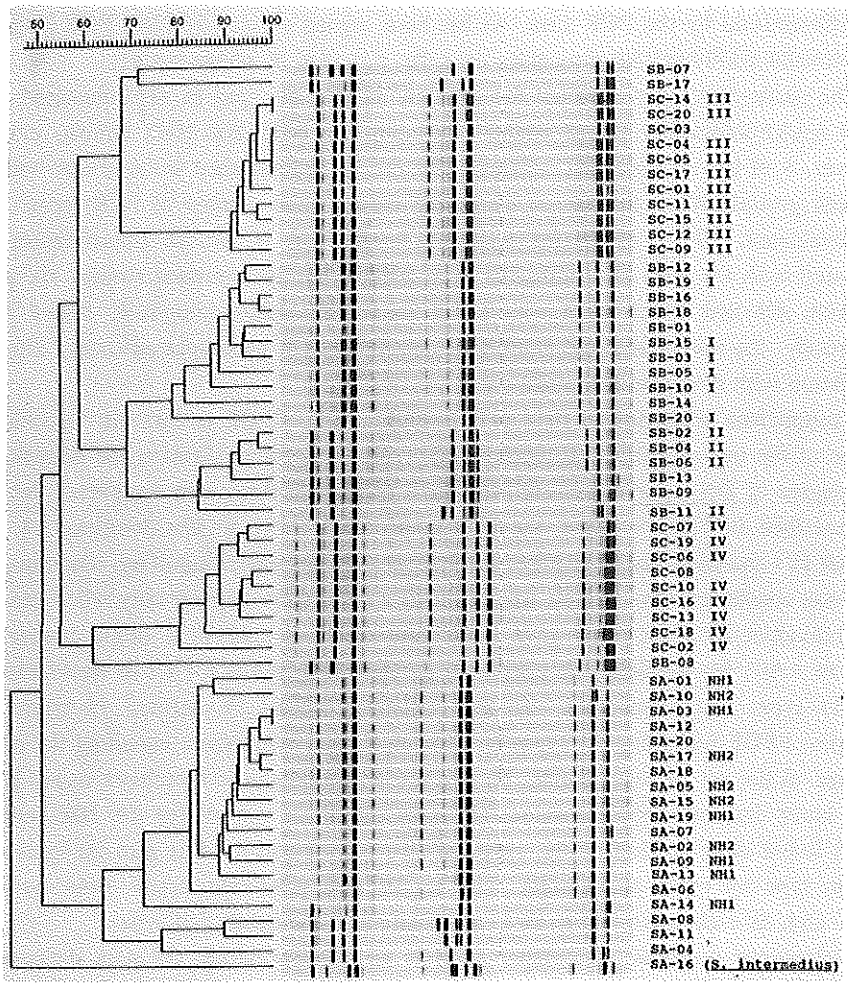


Figure 3 Gelcompar analysis of dataset Ia. The pictures shown in Figure 1 have been digitised by scanning procedures. All three AP PCR DNA banding patterns have been combined into one single lane. Degree of homology was subsequently calculated using Dice comparisons and correlation coefficients were determined by UPGMA analysis. On the right the strain code as presented in Table 1 is shown, as is the deduced three digit genocode (column Ia, Table 1) and the epidemiological clustering.

DISCUSSION

Approximately five years ago, PCR-mediated procedures enabling genome scanning by random amplification of polymorphic DNA were discovered (30, 31). AP PCR can be used for genetic characterisations and comparisons even among closely related bacterial species and isolates (1, 20, 21, 24-26, 28, 29). The procedure is used with increasing frequency, facilitated by general applicability and high speed. However, only a limited number of studies have compared the effectiveness of AP PCR typing with that of other microbiological typing procedures (4, 7, 16, 19-21, 25, 26). In the field of staphylococcal typing, numerous studies describe conventional or molecular elucidation of clonality or epidemiological relatedness. Recently, this was combined in a comparative study on typing of a large panel of *S. aureus* isolates (22).

The overall conclusion from the present data is that AP PCR adequately clusters strains isolated from given outbreaks. On the other hand, considerable differences between the results from different laboratories have been encountered. This is reflected by the number of isolates that are identified by a unique genotype. This number varies from nine to sixteen and participants that detect more than one type among epidemiologically clustered strains score relatively high in this respect. It has to be emphasised that during this study, several of the experimental parameters were standardised. In this respect, it is noteworthy, a relatively high degree of heterogeneity between laboratories was encountered as a result of this limited number of additional variables (Table 2). Including the DNA isolation protocol in the multicenter approach would most probably have led to an even lower degree of interlaboratory reproducibility.

In the single multicenter AP PCR typing study that has been described to date (17), the time- and cost-effectiveness of PCR typing were investigated. This study demonstrated that successful AP PCR depends heavily on the optimal use of PCR protocols (5). For this reason, it was decided not to study the intralaboratory reproducibility of the AP PCR tests. These items have been addressed in previous studies (7, 21, 23-26). However, the fact that epidemiological clusters of strains generate identical DNA-banding patters upon DNA amplification is evidence of at least a reasonable degree of intralaboratory reliability. Upon reamplification of some of the DNA samples, as performed in two of the participating laboratories, AP PCR profiles appeared to be reproducible.

Reproducibility was affected by the nature of the primer used and the identity of the intratube thermoprofile. Isolates SA-04, SB-07 and SC-03, all of which were *S. aureus* type strain ATCC 12600, were included in the three sets of strains to evaluate the reproducibility of AP PCR. Only one of the participating laboratories unequivocally identified all three strains to belong to a single genotype. It must be emphasised that the other typing procedures also detected gross differences among these three particular isolates. It has been demonstrated previously that genetic variability as measured by PCR can be a consequence of repeated conservation and "reviving" of strains; this is probably due to replication defects or the absence or presence of lytic phages (3). This might be an

explanation for the extensive variability encountered among the ATCC strains. PFGE, for instance, corroborates the PCR data from 6 of 7 PCR datasets by designating genotypes E, D and C. Plasmid types are also very different: B, D and C are the indexes. This, in combination with other experimental results, may be indicative of intrastrain heterogeneity or sampling error. Computerised correction of AP PCR artifacts is currently under development (13, 14). It is particularly important to implement this approach, which takes reproducibility and erroneous amplification into account, when multi-center studies are performed. However, on the basis of the results of the present study, it is expected that interinstitute standardisation will be very hard to achieve.

PCR typing is currently restricted to laboratories with appropriate equipment and experimental expertise. In this respect, the applicability of AP PCR is as yet limited. It is clear from the present study that generation and interpretation of PCR data is likely to vary between laboratories. The percentage of variant types identified can be in the order of 27 to 50%, based on the application of three PCR tests and a single DNA-processing protocol. The variables that still exist between laboratories (Table 2) must be responsible, at least in part, for these large discrepancies. From the duplicate experiments performed in Rotterdam, it was concluded that gel electrophoresis is a major cause of experimental variability; this has been confirmed by a recent report (10). An increase in electrophoresis time led to improved separation, which in turn enabled successful digitisation and Gelcompar analysis. It is also acknowledged that the present study suffers from the fact that DNA isolation and primer quality were standardised. If this had not been the case, differences between laboratories may have been even larger.

AP PCR shares characteristics with the genome-scanning capacities of electrophoretic techniques such as PFGE and FIGE. These last two procedures identify epidemiological relations for staphylococci that are in good agreement with the present data (Table 4). Recently, guidelines for interpretation of PFGE patterns for outbreak investigations were proposed by an American working group (8). Since these rules have been used to define staphylococcal subtypes in the collection used in the previous and present studies (22), our PCR data may enable the development of similar rules for the definition of PCR subtyping. The rules should be either based on differences within the banding pattern generated during a single PCR or on differences in composite genocodes as presented in Table 1 and simplified in Table 4. The present study indicates that differences in the combined results of multiple AP PCR assays are better indicators of genetic variability than the results of individual assays. It is clear, however, that PFGE subtypes do not fully match the subtypes as defined by PCR. It is advisable to perform model studies starting with PFGE-uniform strains on the one hand and PCR-uniform strains on the other hand. For studies such as these it is also important to start the analysis on a collection comparable to the set of strains described in this study: it should provide a mixture of unique, solitary isolates together with epidemiologically well-defined clusters.

Our data demonstrate that PCR fingerprinting deserves a position among the pro-

cedures that are well suited for the epidemiological analysis of *S. aureus*. The procedure seems particularly appropriate for the high-speed typing of nosocomial isolates. This conclusion was also drawn previously (2), on the basis of theoretical considerations, it was suggested that AP PCR is a cost-effective procedure as well. It is necessary to test multiple primers, since differences in discriminatory power are to be expected. Strain-specific amplicons can be generated quite easily, even among clonally related isolates of *S. aureus*. With the exclusion of subtype numbering for the other typing strategies, AP PCR generates the largest number of individual types. It exceeds the resolution of PFGE, which detects 11 types and 5 subtypes. Only phage typing and RFLP mapping approach the average number of types detectable by AP PCR. It must be emphasised, however, that the generation of excessive numbers of types introduces the possibility that the relationship between the AP PCR data and epidemiological characteristics will start to deteriorate.

Finally, we recommend establishing collections like the one used in this and the previous study (22) for other microorganisms as well. The availability of these strains enables the individual researcher to establish the value of newly developed typing tools or to use these strains as internal controls in typing studies. Well-documented collections or experimental protocols can be used for standardisation of typing procedures (27), an initiative important for the development of international standards on genetic relatedness or clonality among pathogenic microorganisms.

REFERENCES

1. **Akopyanz, N., N.O. Bukanov, T.U. Westblom, S. Krurovich and D.E. Berg** (1992) DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Res. **20**: 5137-5142.
2. **Bingen, E** (1994) Applications of molecular methods to epidemiologic investigations of nosocomial infections in a pediatric hospital. Infect. Control Hosp. Epidemiol. **15**: 488-493.
3. **Brikun, I., K. Suziedelis and D.E. Berg** (1994) DNA sequence divergence among derivatives of *Escherichia coli* K-12 detected by PCR-based RAPD fingerprinting. J. Bact. **176**: 1673-1682.
4. **Brousseau, R., A. Saint-Onge, G. Prefontaine, L. Masson and J. Cabana** (1993) Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. Appl. Environ. Microbiol. **59**: 114-119.
5. **Caetano-Anolles, G., B.J. Bassam and P.M. Gresshoff** (1994) Buffer components tailor DNA amplification with arbitrary primers. PCR Meth. Appl. **4**:59-61.
6. **Centers for Disease Control** (1990) Postsurgical infections associated with an extrinsically contaminated intravenous anaesthetic agent - California, Illinois, Maine, Michigan, 1990. Morbid. Mortal. Weekly Rep. **39**: 426-433.
7. **Elaichouni, A., G. Verschraegen, G. Claeys, M. de Vleeschouwer, C. Godard and M. Vaneechoutte** (1994) *Pseudomonas aeruginosa* serotype O12 outbreak studied with arbitrary primer PCR. J. Clin. Microbiol. **32**: 666-671.
8. **Goering, R.V** (1994) Defining inter-isolate relationships between bacterial

isolates by use of PFGE of DNA macrorestriction fragments. Abstr. Int. Meeting on Bacterial Epidemiological Markers. Cambridge, UK, April 22-25.

9. **Goetz, M.B., M.E. Mulligan, R. Kwok, H. O'Brien, C. Caballes and J.P. Garcia** (1992) Management and epidemiologic analyses of an outbreak due to methicillin resistant *Staphylococcus aureus*. Am. J. Med. **92**: 607-614.
10. **He, G., C.S. Prakash, R.L. Jarret, S. Tuzun and J. Qiu** (1994) Comparison of gelmatrices for resolving PCR amplified DNA fingerprint profiles. PCR Meth. Appl. **4**: 50-51.
11. **Jarvis, W.R** (1994) Usefulness of molecular epidemiology for outbreak investigations. Infect. Contr. Hosp. Epid. **15**: 500-503.
12. **Kloos, W.E. and W.D. Lambe, Jr** (1991) Staphylococcus, p. 222-227. *In* A. Balows, W.J. Hausler, K.L. Herrmann, H.D. Isenberg and H.J. Shadomy (Ed) Manual of Clinical Microbiology, 5th edition, American Society for Microbiology, Washington, D.C.
13. **Lamboy, W.F** (1994) Computing genetic similarity coefficients from RAPD data: the effects of PCR artifacts. PCR Meth. Appl. **4**: 31-37.
14. **Lamboy, W.F** (1994) Computing genetic similarity coefficients from RAPD data: correcting for the effects of PCR artifacts caused by variation in experimental conditions. PCR Meth. Appl. **4**: 38-43.
15. **Maslow, J.N., M.E. Mulligan and R.D. Arbeit** (1993) Molecular epidemiology: the application of contemporary techniques to typing bacteria. Clin. Infect. Dis. **17**: 153-162.
16. **Mazurier, S., A. Audurier, N. Marquet-Van der Meer, S. Notermans and K. Wernars** (1992) A comparative study of randomly amplified polymorphic DNA analysis and conventional phage typing for epidemiological studies of *Listeria monocytogenes* isolates. Res. Microbiol. **143**: 507-512.
17. **Penner, G.A., A. Bush, R. Wise, L. Domier, K. Kasha, A. Laroche, G. Scoles, S.J. Molnar and G. Fedak** (1993) Reproducibility of random amplified polymorphic DNA RAPD analysis among laboratories. PCR Meth. Appl. **2**: 341-345.
18. **Pfaller, M.A., D.S. Wakefield, R. Hollis, M. Fredrickson, E. Evans and R.M. Massanari** (1991) The clinical microbiology laboratory as an aid in infection control. The application of molecular techniques in epidemiologic studies of methicillin-resistant *Staphylococcus aureus*. Diagn. Microbiol. Infect. Dis. **14**: 209-217.
19. **Ralph, D., M. McClelland, J. Welsh, G. Baranton and P. Perolat** (1993) *Leptospira* species categorised by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. J. Bacteriol. **175**: 973-981.
20. **Saulnier, P., C. Bourneix, G. Prevost and A. Andremont** (1993) Random amplified polymorphic DNA assay is less discriminant than pulsed field gelelectrophoresis for typing strains of *Staphylococcus aureus*. J. Clin. Microbiol. **31**: 982-985.
21. **Struelens, M., R. Bax, A. Deplano, W. Quint and A. van Belkum** (1993) Concordant clonal delineation of methicillin resistant *Staphylococcus aureus* by macrorestriction analysis and polymerase chain reaction genome fingerprinting. J. Clin. Microbiol. **31**: 1964-1970.

22. **Tenover, F.C., R. Arbeit, G. Archer, J. Biddle, S. Byrne, R. Goering, G. Hancock, G.A. Hebert, B. Hill, R. Hollis, W.R. Jarvis, B. Kreiswirth, W. Eisner, J. Maslow, L.K. McDougal, J.M. Miller, M. Mulligan and M.A. Pfaller** (1994) Comparison of traditional and molecular methods of typing of *Staphylococcus aureus*. J. Clin. Microbiol. **32**: 407-415.

23. **Van Belkum, A** (1994) DNA fingerprinting of medically important microorganisms using the polymerase chain reaction. Clin. Microbiol. Rev. **7**: 174-184.

24. **Van Belkum, A. and J. Meis** (1994) Polymerase chain reaction mediated DNA fingerprinting in bacterial epidemiology. Clin. Infect. Dis. **18**: 1017-1018.

25. **Van Belkum, A., R. Bax and G. Prevost** (1994) Comparison of four genotyping assays for epidemiological study of methicillin resistant *Staphylococcus aureus*. Eur. J. Clin. Microbiol. Infect. Dis. **13**: 420-424.

26. **Van Belkum, A., R. Bax, P. Peerbooms, W. Goessens, N. van Leeuwen and W. Quint** (1993) Comparison of phage typing and DNA fingerprinting by PCR for discrimination of methicillin resistant *Staphylococcus aureus* isolates. J. Clin. Microbiol. **31**: 798-803.

27. **Van Embden, J.D.A., M.D. Cave, J.T. Crawford, J.W. Dale, K.D. Eisenach, B. Gicquel, P.W.M. Hermans, C. Martin, R. Mcadam, T.M. Shinnick and P.M. Small** (1992) Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardised methodology. J. Clin. Microbiol.**31**: 406-409.

28. **Versalovic, J., T. Koeuth and J.R. Lupski** (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19**: 6823-6831.

29. **Welsh, J., C. Pretzman, D. Postic, I. Saint-Girons, G. Baranton and M. McClelland** (1992) Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* to three distinct phyletic groups. Int. J. Syst. Bacteriol. **42**: 370-377.

30. **Welsh, J. and M. McClelland** (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18**: 7213-7218.

31. **Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey** (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18**: 6531-6535.

CHAPTER XIII
DISCUSSION AND SUMMARY

With the discovery of efficient antimicrobial agents, the first effective means for curing bacterial infections became available. In combination with vaccination strategies, the social and economic burden posed by infectious diseases could be significantly lowered. However, in modern times bacterial pathogens are found that are increasingly capable of evading immune barriers and display resistance towards several (and sometimes even most) of the clinically available antibiotics. Moreover, immune-deficiency or -suppression is gaining in prevalence and use as part of various medical therapies, respectively. This causes many hospitalised patients to become more and more susceptible to invasive bacterial infection. The rapid development of resistance has surprised the medical community in the past decades but has resulted from large scale application of antimicrobial agents. Bacteria proved genetically flexible and various mechanisms of resistance have been elucidated. Resistance development is a major cause of medical concern and several alarming examples received world wide attention in recent years. Several pathogens almost reached that stage enabling them to cause infections to which no cure exists. Multiresistant *Mycobacterium tuberculosis*, first recognised and encountered epidemic-wise in the state of New York (USA), provides a striking example. This pathogen was first discovered among prison inmates from which it spread to the outside world. Other well-known examples are provided by penicillin-resistant pneumococci, vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* strains. Especially this latter type of microorganism has been studied in great detail and molecular analysis has provided insight both in the nature of the resistance genes and their mode of (international) dissemination. It are studies like these that clearly demonstrate the value and necessity of DNA mediated procedures for identifying and tracking multiresistant pathogens. Once the (molecular) basis of the of the epidemic behaviour of bacteria becomes common knowledge, new antibiotics or intervention procedures may be developed and effective control measures can possibly be designed. From molecular typing studies it became apparent that improvement of the strategies used for characterisation of microbes is a necessity. Insight in the mode of transmission or the location of reservoirs is of major medical importance as was exemplified by studies describing the control of nosocomial epidemics of multiresistant bacterial agents.

A clear advantage of molecular typing of microorganisms over the well established, but generally phenotypic procedures, is that genetic markers are more stable, easier to detect in a reproducible manner, not easily affected by most environmental variables and traceable in diverse laboratory settings. Many different genetic approaches for bacterial identification have been brought forward in the past decades, all of which have

specific merits and drawbacks. Aspects of technical complexity, the need for specialised equipment or reagents, the duration of the analyses and financial burden determine whether or not a procedure will be generally applied or more restricted in use. It can be stated that many of the established molecular procedures are rather costly and complex. Simplification, increasing the speed of such tests and being within the reach of the routine microbiology laboratory are prerequisites for improvements in the efficacy of typing procedures.

In this respect the DNA amplification assays such as the polymerase chain reaction (PCR) may provide innovative possibilities. The PCR is a relatively new molecular biological tool for the analysis of DNA molecules, guided by the amplification of (regions of) DNA molecules through short, synthetic oligonucleotides that determine the target region to be amplified. The PCR is being introduced in several microbiological diagnostic procedures, some of which are already commercially available. The presence of sexually transmitted pathogens including the human immunodeficiency virus, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* can be demonstrated in a single specimen of clinical material. This holds great promise for the application of the PCR in the microbiology laboratory. Since PCR diagnostics may be introduced on a relatively large scale, in virology as well as in bacteriology, other applications of the PCR may become within reach of the clinical microbiologist. The availability of appropriate equipment and technical knowhow concerning PCR methodology will stimulate the use of PCR for microbial typing as well. In that case the PCR is used for the overall screening of genomic DNA molecules. By such an arbitrary amplification assay, global characteristics of the DNA molecule under investigation are obtained. Identical DNA molecules will give rise to identical amplification patterns, related DNA molecules will lead to patterns displaying a certain degree of homology, whereas clearly different template DNA molecules will generate completely different patterns. These patterns, generated by multiple DNA fragments derived from regions scattered throughout the entire genome, can be visualised as DNA profiles upon gel electrophoresis. The result can be considered a bacterial barcode that can be used for classification of multiple species and even of strains from within a given species.

In the present thesis this PCR mediated approach, often referred to as arbitrary primed (AP) PCR or random amplification of polymorphic DNA (RAPD) analysis was used in several matters of protozoan, fungal or bacterial concern. This recently developed technique has been validated in comparison with current gold standard procedures and has been applied to clinical-epidemiological problems. Results provided by molecular typing can be used to assess persistence or dissemination of (multi-resistant) pathogens in or between patients, patients and medical personnel and even between institutions or, on a larger scale, between nations or continents (for a general introduction see chapters I and II).

The initial experimental studies were aimed at the determination of clonality

among several species of protozoan parasites (see chapters III and IV). When species and isolates of *Giardia duodenalis* and *Naegleria fowleri* were studied by arbitrary primed PCR it was demonstrated that depending on the protozoan species, the same technique gave rise to different conclusions with respect to genetic heterogeneity and species definition. Apparently, *N. fowleri* behaves as a genetically stable, universal clone, whereas *G. duodenalis* displays enhanced flexibility and variability at the DNA level. Even among clonally related isolates of this latter species, deviating DNA fingerprints could be generated with relative ease. Among the strains in a worldwide collection of *N. fowleri* isolates, only a very limited number of geographically clustered strains displayed some (but identical) variation in the DNA banding patterns as generated by AP PCR. It appears as if *G. duodenalis* evolves at a higher speed than *N. fowleri* does. This can possibly be explained by the nature of the ecological niches in which the parasites exist, the *G. duodenalis* strains being more prevalent in a more variable, immune-pressurised, human environment.

Molecular typing of pathogenic fungi is of importance in nosocomial epidemiology. The possibility to earmark a fungal strain on the basis of genetic characteristics enables local and (inter)national tracking of clonally related strains. In such a way, insight in epidemiology and dissemination can be obtained, which can be of importance for the development of appropriate sanitary measures within individual hospitals. Adequate shielding should prevent the spread of fungal pathogens among susceptible, usually immune-compromised patients. In recent years, the DNA mediated typing procedures found their way into the medical mycology laboratory, in particular since no reliable phenotypic typing procedure is readily available for the analysis of fungal isolates in general. However, for technical reasons even the DNA-mediated procedures are lengthy and cumbersome. Among the better procedures are several PCR-mediated assays. PCR tests can focus on the detection of restriction fragment length polymorphisms (RFLP) or DNA sequence variation in general, as was described for the clinically most prevalent fungal species. Otherwise, PCR can again be used to amplify arbitrary regions of genomic DNA. The latter technique has found acceptance among hospital epidemiologists, medical microbiologists and molecular biologists since it allows the genetic analysis of collections of fungal strains thought to be involved in (nosocomial) outbreaks. Chapters V through VIII provide examples of PCR typing in the field of medical mycology.

Malassezia spp can cause invasive disease, for instance in neonates. For these species it was demonstrated that AP PCR is a more effective typing procedure than pulsed field gel electrophoresis (chapter V). Furthermore, on the basis of a longitudinal survey in a neonatal intensive care unit, it was demonstrated that strains of this skin-inhabiting yeast can persist on the surface of incubators for prolonged periods of time, despite regular cleaning and disinfection. Apparently, removing *Malassezia* spp from plastic or glass surfaces requires aggressive decontamination.

For the two major fungal pathogens, *Aspergillus fumigatus* (see chapter VIII) and

Candida albicans, several studies demonstrated the feasibility of AP PCR mediated tracking of clonal dissemination and analysis of the dynamics of long-term colonisation. For *C. albicans* two large longitudinal screening studies among neutropenic patients were performed with the aid of AP PCR (chapters VI and VII). Among children undergoing bone marrow transplantation it was demonstrated that all sorts of medical treatment, including antifungal therapy, did not affect the nature of their colonising *C. albicans* strain. The genetic identity of isolates could be documented for strains collected over periods as long as half a year from individual patients. This demonstrates the genetic homogeneity of the *C. albicans* populations colonising young human individuals. Furthermore, cross-colonisation between patients in neighbouring wards was never observed indicating the adequacy of the infection prevention measures aimed at the prevention of dissemination of this opportunistic pathogen. Among adult neutropenic patients, being on fluconazole prophylaxis, it was shown that in 29% of cases a new strain was incidentally cultured, which shows that upon ageing humans acquire multiple strains of *C. albicans*. In the single instance in which fluconazole resistance was developed, no genetic differences between the sensitive and resistant isolates could be documented. This indicates resistance development within a given strain instead of selection of another, naturally resistant one, proving that under environmental pressure clonal populations of *C. albicans* can evolve into specific directions.

In the last four chapters (IX through XII) the application of AP PCR for genetic analyses of *Staphylococcus aureus* is described. Since *S. aureus* is an important opportunistic pathogen, especially in the hospital setting, many epidemiological studies and consequently many experimental strategies for studying this bacterial species have been described in the literature. For this reason the PCR studies on *S. aureus* were mainly technical in nature, aiming at assesment of the epidemiological value of the PCR data. From a clinical perspective, studies focused on unravelling the genetic relatedness of the methicillin-resistant variants of the golden staphylococcus. It was initially demonstrated that the gold standard procedure for staphylococcal analysis, phagetyping, displayed a lesser discriminatory power than AP PCR (chapter X). Epidemiological relatedness, however, could be reliably documented by the PCR approach. In a subsequent study it was demonstrated that PCR techniques can withstand the comparison with other DNA mediated typing approaches as well. Among four different techniques, which included ribotyping and pulsed field gel electrophoresis of DNA macrorestriction fragments, PCR appeared to be both technically and economically attractive, although the resolution of the procedure depended on the nature and number of primer(s) used (chapter XI). In a multicentered study, however, one of the major disadvantages of AP PCR was highlighted (chapter XII). Sixty well-characterised strains of *S. aureus* were subjected to PCR-mediated typing. A single DNA batch was obtained for all isolates and portions were distributed to a number of microbiological laboratories, this in combination with appropriate PCR primers and a detailed technical protocol. Although data generated in different

centers were mostly in agreement with epidemiological data (and data obtained by other typing procedures) the reproducibility between centers was not very good. This implied that AP PCR is very well suited for local applications in matters of infectious concern, but that for large inter-institutional studies the method should be rigorously standardised. The general usefulness and clinical relevance of AP PCR, also for typing of methicillin-sensitive *S. aureus*, was demonstrated in a study in a paediatric ward in a general hospital where several children presented with staphylococcal infections (chapter IX). It was proven that virulent staphylococcal clones circulated and that, more importantly, specific DNA probes useful for the detection of these virulent staphylococcal clones, could be isolated as a side product of the typing procedure.

The examples mentioned above clearly indicate the usefulness and versatility of PCR mediated typing for molecular tracking of microbial pathogens. As such, the PCR approach deserves a place among the other, more accepted DNA typing procedures applied for microbial-ecological purposes. However, since the technique is relatively new and is still open to further improvement, especially from the technical point of view, future studies are needed. These should be aimed at multicentered evaluation of the AP PCR tests and on standardisation of data processing and inter-laboratory data exchange. Furthermore, and certainly not least in importance, the PCR mediated strategy enables the straightforward isolation of genetic markers, aimed at genus, species and even strain level. Sets of the latter type of probes may be useful for binary typing of microbes, whereas direct mapping or isolation of genetic traits including those for virulence or resistance against antibiotics is feasible as well.

Finally, besides the application of the PCR mediated procedures within the field of clinical microbiology, the same techniques can also be used for finding answers to more basic questions concerning microbial evolution and population dynamics. These phenomena can be studied in the long term or a short term fashion. By randomly screening genomes for point mutations and events related to the acquisition, loss, moving or multiplication of intra- or extra chromosomal genetic material, regions displaying an enhanced rate of molecular evolution can be identified. As such, the speed with which (parts of) the genome of microorganisms evolve can be determined and the molecular or environmental background of these phenomena can be studied in detail. Hypervariable region or elements can be tracked down which may have implications for the construction of "environmentally safe" genetically modified microorganisms. Establishing the evolutionary clockspeed of DNA regions or elements will also affect the interpretation of data generated in the context of nosocomial epidemiology. Research on microbial evolution within the field of bacteriology may eventually lead to quasi species concepts, this in parallel with ongoing discussions in the field of human and animal virology. The urgency of this type of investigations is clearly indicated by the high speed emergence and spread of successful bacterial clones, traveling the entire world and confronting the human host with increases in morbidity and mortality.

SAMENVATTENDE DISCUSSIE

Eerder deze eeuw werd met de ontdekking van bruikbare antimicrobiële middelen een effectieve bestrijding van bacteriële infecties mogelijk gemaakt. Mede door het parallel gebruik van vaccins kon de sociale en economische last die door infectieziekten werd veroorzaakt drastisch omlaag gebracht worden. Helaas zijn de huidige bacteriële pathogenen op een bedreigende wijze in staat om zowel de effectieve werking van antibiotica alsmede die van het werkend immuunsysteem te omzeilen. Met name op plaatsen waar veelvuldig antibiotica toegediend worden en waar patiënten met immuunstoornissen prevalent zijn wordt een toenemende infectiedruk waargenomen. Dit heeft tot gevolg dat met name de ziekenhuispatiënt in toenemende mate geconfronteerd wordt met infectiegevaar.

De snelheid waarmee een breed spectrum aan bacteriële pathogenen resistentie tegen een groot aantal antibiotica heeft verworven wordt door klinici en medisch microbiologen als zeer bedreigend ervaren. Bacteriën die onder antibiotica druk verkeren blijken zeer flexibel in het ontwijken van een effectieve therapie. Heden ten dage is al een veelheid aan moleculaire mechanismen die ten grondslag liggen aan bacteriële resistentie tot in detail beschreven. Deze zorgwekkende ontwikkelingen hebben internationaal veel aandacht getrokken, zeker gezien het feit dat infecties met een aantal van de multiresistente pathogenen nauwelijks meer behandelbaar zijn. De multiresistente tuberculose verwekker, *Mycobacterium tuberculosis*, werd recent voor het eerst op vrij grote schaal aangetroffen in het Amerikaanse New York. De gevaarlijke ziekteverwekker werd gevonden binnen een groep gevangenen, maar al snel kon ook verspreiding buiten de penitentiaire instelling waargenomen worden. Andere bekende voorbeelden zijn penicilline-resistente pneumokokken, vancomycine-resistente enterokokken of meticilline-resistente *Staphylococcus aureus* (MRSA) stammen. Met name aan MRSA is veel onderzoek verricht: met behulp van moleculaire technieken is inzicht verworven in de basale details van het resistentie mechanisme en in de manier waarop de bacterie zich (internationaal) verspreid lijkt te hebben. De laatste klasse van onderzoekingen heeft aangetoond wat de waarde is van een verbeterd inzicht in de wijze waarop een pathogeen zich verspreid en weet te handhaven. Als de verspreidingswijze eenmaal duidelijk is kan mogelijk met (restrictief) gebruik van nieuwe antibiotica of totaal andere interventieprocedures aan infectiecontrole gewerkt worden. Dat verbetering van deze controle noodzakelijk is, is uit (moleculaire) typeringsstudies naar voren gekomen. Naast het verbeteren van het inzicht in verspreidingsmechanismen is ook het aantonen en in kaart brengen van reservoirs van eminent medisch belang.

Moleculaire typering van bacteriële pathogenen heeft een aantal voordelen ten opzichte van de klassieke, vaak meer fenotypisch georiënteerde strategieën. Genetische kenmerken zijn over het algemeen stabieler dan de uiterlijke kenmerken, ze zijn makkelij-

ker en op een meer reproduceerbare wijze te bepalen, variëren niet afhankelijk van de omgevingskarakteristieken en kunnen in diverse typen laboratoria (afhankelijk van traditie en expertise) bepaald worden. Er zijn in de afgelopen jaren veel genetische typeringsprocedures ontwikkeld, elk met specifieke voor- en nadelen. Zaken als technische complexiteit, behoefte aan specifieke ingrediënten of apparatuur, snelheid van de analyse en kostenaspecten bepalen de breedte waarin een procedure toepasbaar is. Samenvattend kan echter gesteld worden dat de moleculaire procedures kostbaar en technisch complex zijn. Om hier verbetering in te brengen is er behoefte aan vereenvoudiging van de protocollen, verhoging van de snelheid en brede introductie in het algemene medisch-microbiologisch lab.

Het is op het laatste vlak dat met name nucleinezuur amplificatie technieken zoals de polymerase ketting reactie (polymerase chain reaction, PCR) innovatieve mogelijkheden bieden. De PCR is relatief nieuw en biedt de mogelijkheid om DNA moleculen tot in detail in kaart te brengen. Geleid door korte synthetische DNA moleculen (oligonucleotiden, in dit verband ook wel primers genaamd) kunnen stukjes van een groot molecule tot grote hoeveelheden vermeerderd worden. Van belang is dat de PCR in de vorm van commercieel verkrijgbare diagnostische testen nu reeds geïntroduceerd wordt in het klinisch microbiologisch laboratorium. De beschikbaarheid van commerciële PCR testen voor bijvoorbeeld de sexueel overdraagbare pathogenen *Chlamydia trachomatis*, *Neisseria gonorrhoeae* of het humane immunodeficientie virus HIV houdt grote beloftes in voor de klinisch bacterioloog of viroloog. In een enkel klinisch specimen kunnen meerdere pathogenen via dezelfde, parallelle technologie aangetoond worden. Juist omdat diagnostische PCR op een vrij grote schaal geïntroduceerd zal gaan worden, zullen ook de andere toepassingsmogelijkheden van de PCR binnen het bereik van de klinisch microbioloog komen. Beschikbaarheid van apparatuur en het in huis hebben van specifieke kennis zal het gebruik van de PCR voor microbiële typering ook stimuleren. Hierbij wordt de PCR toegepast op een wijze die totale variatie-analyse van een DNA molecule mogelijk maakt. Resultaat is dat DNA van identieke stammen van een bepaald pathogeen tot een identiek amplifikatiepatroon aanleiding zullen geven. DNA van aan elkaar verwante stammen zal tot sterk gelijkende, maar niet-identieke patronen aanleiding geven, terwijl duidelijk verschillende microbiële stammen niet-gelijkende patronen genereren. De profielen, opgebouwd uit geamplificeerde DNA moleculen van verschillende lengte, kunnen zichtbaar gemaakt worden met behulp van gel electroforese en fluorescente kleuring. Het experimenteel resultaat kan beschouwd worden als een genetische barcode die gebruikt kan worden voor identificatie van zowel species als stammen van binnen een enkele microbiële soort.

In het onderhavige proefschrift wordt beschreven hoe de PCR in een protocol dat meestal omschreven wordt als "arbitrary primed" (AP) PCR of "random amplification of polymorphic DNA" (RAPD) analyse, toegepast kan worden binnen vraagstellingen van microbiologische aard. De PCR techniek is uitgebreid vergeleken met andere typerings-

procedures en is aangewend om klinisch-epidemiologische problemen te benaderen. De typeringsresultaten kunnen gebruikt worden om inzicht te verkrijgen in persistentie of verspreiding van (multiresistente) microorganismen in of tussen patiënten en / of medisch personeel. Verder kan met dezelfde procedures inzicht verkregen worden in verspreiding tussen instituten, landen of continenten (voor een schets van het perspectief en een literatuuroverzicht, zie hoofdstukken I en II).

De eerste experimentele studies waren gericht op het aantonen van relaties tussen en binnen verscheidene groepen van protozoaire parasieten (Hoofdstukken III en IV). Wanneer isolaten van verschillende *Naegleria* spp of *Giardia duodenalis* werden bestudeerd met AP PCR, bleek dat afhankelijk van het soort met meer of minder gemak genetische heterogeniteit binnen een species aangetoond kon worden. Waar *Naegleria fowleri* zich leek te gedragen als een genetisch stabiel microorganisme (een universele kloon) bleken stammen van *G. duodenalis* op DNA niveau veel sterker te variëren. Zelfs zeer nauw aan elkaar verwante stammen van het laatste organisme bleken verschillende AP PCR karakteristieken te genereren. Voor *N. fowleri* geldt dat slecht voor een beperkt aantal isolaten uit eenzelfde geografisch geïsoleerde oorsprong een beperkte variabiliteit vastgelegd kon worden. Het lijkt er dientengevolge op dat het genoom van *G. duodenalis* met een hogere snelheid evolueert dan het genoom van *N. fowleri*. Dit zou bepaald kunnen worden door de ecologische niche waarin de protozoaire parasieten voornamelijk voorkomen: *G. duodenalis* floreert in de humane gastheer, alwaar de parasiet het hoofd moet bieden aan immunologische afweer, terwijl *N. fowleri* dat in mindere mate hoeft te bewerkstelligen.

Moleculaire typering van gisten en schimmels is in het kader van infectiepreventie en medische epidemiologie van belang. De mogelijkheid om een fungus aan de hand van het DNA te karakteriseren maakt het vervolgen van lokale en soms zelfs (inter)nationale verspreiding zeer wel mogelijk. Zodoende wordt inzicht verworven in de manier waarop gisten en schimmels kunnen dissemineren, wat weer aanleiding kan geven tot verbetering van hygiënische of afschermingsmaatregelen. Met name immuungecompromitteerde patiënten vormen een belangrijke risicogroep. Vanwege het gebrek aan goede fenotypische methoden, biedt de DNA technologie juist voor schimmels nieuwe ongekende mogelijkheden. Problematisch is echter het tijdrovend karakter van de meeste DNA-gemedieerde procedures. De PCR methoden vormen hierop echter een uitzondering. De snelheid ligt hoog en er kan gericht worden op AP PCR procedures, maar ook op de detectie van variabele restrictie enzym herkenningsplaatsen. Ziekenhuisepidemieen van gist- of schimmelinfecties worden momenteel veelvuldig met behulp van de PCR bestudeerd. Hoofdstukken V tot en met VIII bieden voorbeelden uit het veld van de medische mycologie.

Malassezia spp kunnen, bijvoorbeeld bij pasgeborenen, invasieve infecties veroorzaken. In hoofdstuk V wordt aangetoond dat in geval van dit species met behulp van AP PCR op een goede wijze tussen isolaten gediscrimineerd kan worden. AP PCR

voldoet in dit opzicht beter dan bijvoorbeeld pulsed field gel electroforese. Verder kon met behulp van AP PCR aangetoond worden dat de van nature op de huid voorkomende gistsoorten *Malassezia furfur* en *Malassezia pachydermatis* ook in staat zijn om het oppervlak van couveuses langdurig te koloniseren. Dit ondanks het gegeven dat diezelfde oppervlakten regelmatig met detergentia schoon werden gemaakt. Blijkbaar dient agressief gedecontamineerd te worden. Dezelfde stammen die op het couveuse oppervlak werden aangetroffen bleken ook infecties in neonaten te kunnen veroorzaken.

Reeds in meerdere studies is de bruikbaarheid van AP PCR voor het volgen van de twee klinisch meest gediagnostiseerde fungi, *Candida albicans* en *Aspergillus fumigatus*, aangetoond. Ook lange termijn kolonisatie kan met AP PCR vervolgd worden. Hoofdstukken VI and VII beschrijven twee lange-termijn studies zoals uitgevoerd onder verschillende groepen van immuun-gecompromitteerde, neutropene patiënten. Voor kinderen die beenmerg transplantatie moesten ondergaan werd aangetoond dat gedurende het gehele behandelingstraject de aard van de koloniserende *C. albicans* niet veranderde. Genetische identiteit van een verscheidenheid aan klinische isolaten bleek soms voor perioden van meer dan een half jaar. Blijkbaar worden jonge individuen slechts door een enkele stam persistent gekoloniseerd. Verder werd binnen deze patiëntengroep nooit kruis infectie / kolonisatie waargenomen, ook werden er vanuit omliggende medisch-oncologische afdelingen nooit *C. albicans* stammen geïmporteerd. Onder neutropene volwassenen werd in een verhoogde frequentie (in 29% van de gevallen) incidenteel een afwijkende stam geïsoleerd. Dit duidt erop dat tijdens veroudering kolonisatie met meerdere stammen tot de mogelijkheden gaat behoren. Alle betrokken patiënten werden serieel met fluconazol behandeld. In één geval ontwikkelde de koloniserende stam resistentie tegen dit antifungale middel, iets wat erop wijst dat dit geen frequent optredende ontwikkeling is. In het ene geval dat dit wel het geval bleek te zijn, wees genetische identiteit tussen het gevoelige en het resistente isolaat erop dat geen rekolonisatie met een andere, reeds resistente stam had opgetreden. Hiermee werd aangetoond dat een residente populatie gistcellen onder antibioticumdruk kan evolueren tot een homogene, resistente gemeenschap.

In de laatste vier hoofdstukken (IX tot en met XII) wordt de toepassing van AP PCR voor genetische analyse van *Staphylococcus aureus* beschreven. Omdat *S. aureus* een belangrijk ziekenhuis-pathogeen is, is er in het verleden vaak aandacht aan epidemiologische studies van deze bacteriesoort gegeven. Een groot aantal technieken is in dit opzicht waardevol gebleken. Vanwege deze redenen zijn de hier beschreven studies in belangrijke mate technisch van aard: van primair belang was het vaststellen van de bruikbaarheid van de AP PCR technologie. Vanuit klinisch perspectief was het van belang om ook voor de meticilline resisten versie van *S. aureus* de bruikbaarheid van AP PCR aan te tonen. Initieel werd aangetoond dat de AP PCR de vergelijking met de gouden standaard typeringsprocedure voor *S. aureus*, de zogenaamde faagtypering, goed kon doorstaan (hoofdstuk X). De resolutie van de PCR aanpak was uitstekend, epidemiologi-

sche gerelateerdheid bleek daarnaast in stand gehouden te worden. In een volgende studie bleek dat de AP PCR ook de vergelijking met andere genetische typeringsstrategien goed doorstond. In vergelijking met procedures als het klassieke ribotyperen en macrorestrictieanalyse met behulp van pulsed field gel elektroforese bleek de AP PCR zowel technisch als economisch zeer aantrekkelijk. Hierbij dient opgemerkt te worden dat de resolutie van de AP PCR analyse wel sterk primer afhankelijk bleek (hoofdstuk XI). In een multicenterstudie (hoofdstuk XII) bleek echter een belangrijk probleem van de AP PCR. Negenenvijftig goed gekarakteriseerde *S. aureus* stammen werden in zes verschillende laboratoria getypeerd volgens een gestandaardiseerd AP PCR protocol. DNA werd in één van de laboratoria geïsoleerd en in porties onder de andere deelnemers verspreid, dit in combinatie met primers en een experimenteel protocol. De data waren in het algemeen redelijk tot zeer goed in overeenstemming met epidemiologische gegevens en de uitslagen van de andere, eerder uitgevoerde typeringsanalyse: epidemiologisch verwantschap werd in alle laboratoria betrouwbaar vastgelegd. Echter, de experimentele gegevens, de DNA banderingspatronen, bleken van lab tot lab vrij sterk te verschillen. Dit leidde tot de conclusie dat de AP PCR lokaal een aan te bevelen procedure is. Voor grote, vergelijkende studies waarbij verschillende labs dezelfde techniek gebruiken en in een later stadium de data willen vergelijken dient de AP PCR techniek nog vergaand gestandaardiseerd te worden. De lokale bruikbaarheid van de AP PCR procedure werd nog eens extra aangetoond in een studie onder neonaten in een perifeer ziekenhuis (hoofdstuk IX). Het bleek dat de circulatie van een virulente *S. aureus* kloon aantoonbaar was en, misschien nog wel belangrijker, het bleek dat met behulp van AP PCR DNA probes die geschikt waren voor het aantonen van diezelfde virulente stafylokokken gegenereerd konden worden.

De hiervoor genoemde voorbeelden geven duidelijk aan dat AP PCR een methode is die het vervolgen van bacteriële pathogenen en medisch relevante fungi in een klinische omgeving zeer wel mogelijk maakt. In dit opzicht verdient de AP PCR een prominente plaats onder de reeds meer geaccepteerde, andere genetische typeringsprocedures. Voordat de AP PCR echter in brede surveillance studies (met name die waarin meerdere laboratoria participeren) toegepast kan gaan worden, dienen additionele studies naar de standaardisatie van de procedure ondernomen te worden. Vooral het verwerken en uitwisselen van de experimentele gegevens dient hierbij aandacht te krijgen. Verder kunnen met behulp van AP PCR genetische markers geïsoleerd worden. Deze markers kunnen specifiek zijn voor genera, species en soms zelfs isolaten van een species. Met name deze laatste klasse van DNA probes kunnen in de nabije toekomst misschien gebruikt gaan worden voor directe analyse van kenmerken als virulentie of resistentie tegen bepaalde antibiotica. Verder kan met behulp van deze isolaat-specifieke probes een binair typeringssysteem voor alle klassen van microbiologisch relevante pathogenen opgezet worden.

Tenslotte, naast de toepasbaarheid binnen de klinische microbiologie, zijn de PCR

technieken tevens toepasbaar in meer basaal georiënteerde onderzoeksvelden. Zowel korte termijn als lange termijn evolutie van bacteriën is voor gedetailleerd onderzoek toegankelijk. Gerandomiseerde PCR analyse van genomische DNA moleculen kan leiden tot de precieze identificatie van gebieden waar puntmutaties hebben opgetreden of waar acquisitie, verlies, verplaatsing of vermenigvuldiging van intra- of extra-chromosomale elementen in het geding is. Zodoende kan de snelheid waarmee (delen van) een bacterieel genoom veranderd (veranderen) bepaald worden en kan inzicht verkregen worden in de moleculaire achtergrond van dit fenomeen. Bekendheid met variabele versus meer constante gebieden in het genomisch DNA van bacteriën zou in de praktijk gebruikt kunnen worden voor de ontwikkeling van "veilige", met behulp van recombinant DNA technieken geconstrueerde microorganismen. De evolutionaire kloksnelheid, zoals met verschillende technieken op een verschillend niveau wordt bepaald, zal tevens implicaties hebben voor de manier waarop binnen de ziekenhuisepidemiologie moleculaire data geïnterpreteerd zullen gaan worden. Naast het binnen de humane en veterinaire virologie veelbesproken concept van de virale quasi-species, zullen in parallel met dit concept ook voor bacteriële pathogenen vergelijkbare beschouwingen, ondersteund met gedetailleerde typeringsdata, opgezet kunnen worden. De zin van evolutionair onderzoek aan bacteriële pathogenen wordt onderstreept door het speelse gemak waarmee succesvolle bacteriële kloons zich over de totale aardbol weten te verspreiden. Deze verspreiding gaat in een aantal gevallen gepaard met verhogingen in morbiditeit en mortaliteit onder humane individuen.

DANKWOORD

Alle mensen die op de één of andere wijze bij hebben gedragen tot de wording van dit boekje staan voor het merendeel met naam en toenaam vermeld aan het begin van één of meerdere van de hoofdstukken. Daarnaast dienen nog een beperkt aantal andere personen genoemd te worden. Ditty de Keizer is degene geweest die van mijn handmatige krabbels leesbare versies van de meeste hoofdstukken heeft gefabriekt. Met weemoed denk ik aan deze secretariële ondersteuning terug. Marjan Bumkens was degene die in eerste instantie opperde "om eens iets met MRSA te gaan doen". Zij zal zich deze suggestie, tussen neus en lippen geopperd tijdens een werkbespreking, misschien niet eens meer herinneren, voor mij is het een duidelijk startpunt geweest. Huub Schellekens heeft een aantal van de manuscripten op een kritische en voor mij leerzame wijze van zijn kanttekeningen voorzien. Aan het thuisfront hebben Aukje, Iefke, Renske en Erna een grotere bijdrage dan die van alle hierboven genoemde mensen aan de tot stand koming van dit boekje geleverd.

CURRICULUM VITAE

Alex van Belkum

Home Address: Cobetstraat 42
2313 KD Leiden
The Netherlands

Date, Place of Birth: June 17, 1959; Warmond, The Netherlands

Nationality: Dutch

Education:

1977	Graduation Atheneum B Scholengemeenschap Bonaventura-Kijckenborg Leiden, The Netherlands.
1983	Doctoral examination Biochemistry, University of Leiden, The Netherlands.
1988	Ph.D. examination Molecular Biology, University of Leiden, The Netherlands.
1989	Course on the handling of experimental animals (Dutch Art.9 License) Veterinary Faculty, University of Utrecht, The Netherlands.

Previous and Present Positions:

1983-1987	Scientific Assistent, Department of Chemistry, Laboratory of Biochemistry, University of Leiden, The Netherlands.
1987-1988	Visiting Scientist, Department of Chemistry, Laboratory of Biochemistry, University of Leiden, The Netherlands.
1988-1990	Research Scientist, Institute for Applied Radiobiology and Immunology, Primate Center TNO, Department of Chronic and Infectious Diseases, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands.
1990-1991	Research Scientist, Medscand Ingeny B.V., Zernikedreef 9, 2300 AR Leiden, The Netherlands.
1991-1994	Staff member Department of Molecular Biology, Diagnostic Centre SSDZ, General Hospital "Reinier de Graaf Gasthuis", Reinier de Graafweg 7, 2600 GA Delft, The Netherlands.
1994-present	Staff member Institute of Clinical Microbiology and Antimicrobial Therapy, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam; Academic Hospital Dijkzigt, Department of Bacteriology, Dr.Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

Rehske


Jefke

Aukje