Yeast molecular identification and typing

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Definitions

- Clone: group of genetically related isolates which are derived from a common parental ancestor being not distinguishible among them by different biochemical, morfological and molecular typing techniques.
- Strain: isolate or group of isolates that can be differentiated from other belonging to the same species when considering phenotypic and/or genotypic characteristics.
- Colonization: first stage of microbial infection. Establishment of the pathogen at the appropriate portal of entry. Pathogens usually colonize host tissues that are in contact with the external environment. Sites of entry in human hosts include the urogenital tract, the digestive tract, the respiratory tract and the conjunctiva.
- Infection: invasion and multiplication of an infectious agent in body tissues, which may be clinically unapparent or result in local cellular injury due to competitive metabolism, toxins, intracellular replication or antigen antibody response. The infection may remain localised, subclinical and temporary if the body defensive mechanisms are effective. A local infection might persist and spread by extension to become an acute, subacute or chronic clinical infection or disease state. A local infection may also become systemic when the microorganisms gain access to the lymphatic or vascular system.
- PCR: Polymerase Chain Reaction. Technique for amplifying DNA sequences *in vitro* by separating the DNA into its two strands and incubating it with oligonucleotide primers and DNA polymerase. It can amplify a specific sequence of DNA by as many as one billion times That is important in biotechnology, forensics, medicine, and genetic research.
- Sensitivity: The sensitivity of a diagnostic method is the proportion of cases analyzed that are positive and are detected as positive.
- Specificity: The efficiency of a diagnostic method is the proportion of cases analyzed that are negative and are detected as negative.

Introduction

Yeast infections are an important cause of morbility and mortality in critical ill patients. The rapid detection of the presence of a yeast in blood and other tissues is a crucial objective that is currently attacked by applying different molecular methods. The detection of nucleic acids in the clinical sample constitutes an alternative that is being deeply investigated for the diagnosis of invasive mycosis. Comercial tests for this purpose are still not available. As a consequence of the restricted sensitivity and especificity of classical microbiology methods, which moreover are time consuming, several molecular biology approaches have won a great potential within the mycology diagnostic area during last decade. Molecular methods based on the study of yeast nucleic acids had their first attemps on the analysis of complementary of nuclear DNA, determining the existance of cospecific relation between strains whose

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morfologic and physiologic characteristics were different even being considered interspecific varying traits (e.g. the genus *Torulopsis*, which lacks pseudohifas, is cospecific with *Candida*, wich dorms these structures; *Hansenula* assimilates nitrates and is coespecific with *Pichia* which does not) (Kurtzman, 1994).

Firstly, different isolates belonging to the same species were distinguished by biotyping or serotyping . Now, using molecular typing methods, going further than biotype or serotype is posible, being even possible to identify if different isolates belong to the same strain (Faber, 1996).

Genotyping includes the direct analysis of chromosomal or extrachromosomal DNA and it has many advantages over the classical typing methods based on phenotypic characteristics (Versalovic *et al*, 1993; Faber, 1996). Without doubt, the highest advancement that genotyping brings is a powerful and trustworthy discriminatory capacity, which permits to differentiate two phylogenetically close related strains (Farber, 1996).

Yeast infections do not use to cause defined clinical manifestations that permit to suspect them (Flahaut *et al*, 1998; Fugita *et al*, 1995). This non concise clinic joined to the lack of a fast and sensitive diagnostic method makes difficult an adequate response against the process. Classical methods based on culture procedures have an estimated sensitivity of 50% (Einsele *et al*, 1997) and they need at least 48 h to proportionate a diagnose (Holmes *et al*, 1994). Thus, the development of an efficient, quick and sensitive diagnostic method would allow the prescription of an early antifungic treatment, what would finally mean a better prognostic. On the other hand, most Candidiasis have an endogenous origin, i.e. produced by yeast already present in the body (Garcia-Ruiz *et al*, 2004). Then, a first objective is the development of fast diagnostic methods, being necessary the ulterior differentiation between colonization and infection.

Since the 90s, several molecular diagnostic methods have been developed and assayed, trying to detect specific genomic sequences of *Candida*, in order to get a feasible, fast, efficient and sensitive diagnose of invasive candidiasis.

Methods based on the Polimerase Chain Reaction (PCR) clearly show a higher sensitivity than culture and a comparable efficiency. Several PCR protocols yield the detection and the identification of *Candida* species in less than 24 h (Chang *et al*, 2001; Shin *et al*, 1997). However, standardization of the extraction and purification of DNA, as well as of the PCR products, is still not well established. At present, Real Time PCR with fluorescence emission seems to be the most promising alternative. It adds the advantages of quantification of the fungi charge and minimization of sample handling and then the risk of false positive results.

Among the most extended techniques, PCR amplification is remarkable by its high sensitivity and quickness. This technique has been implemented in various approaches: PCR with specific primers at the genus or species level, Multiplex PCR, PCR amplification and subsequent Southern blot, PCR and posterior restriction analysis of amplicons, nestled PCR, Real Time PCR, PCR and later sequencing (Loeffler *et al* 2001; Ahmad *et al*, 2002; Alcoba-Florez *et al*, 2005).

With respect to the detection of fungi as causal agents of infection in different clinical samples, the most studied targets are the gene encoding the 18S rRNA and the adjacent ITS (internal transcribed spacer), sequences included in the ribosomal RNA operon. This operon presents highly conserved regions, what brings out the possibility of detecting and identifying fungi at the genus level by the so called panfungi PCRs. The operon also includes hypervariable regions differing in sequences and/or length, regions that permit identification at the species level. This identification can be achieved by different approaches as for example PCR probes and hybridyzation, Real Time PCR, capillary electrophoresis of amplicons, sequencing, etc. One of the first studies using this type of approach demonstrated its utility in patients with episodes of fever, neutropenia and possible invasive fungemia. The analytic sensitivity in blood was 1 cfu/ml. PCR was demonstrated to be the earliest method to indicate fungic infection, preceding the clinic and radiological evidences, and correlating with a rapid positive response to therapy and clinical evolution (Verweij P *et al*, 2000). The highest value of PCR is its high negative prediction power, which would permit to early discharge the existence of fungic infection, reducing then the empiric use of antifungics (Hebart *et al* 2000; Williamson *et al*, 2000). However, these techniques should be still considered under investigation and then are not part of the

diagnostic criteria for invasive mycosis. For the routine use of the molecular methods herein discussed some technical aspects have to be further improved, as for example the fungic DNA extraction methods and the controls to avoid false positives because of contamination with environmental fungi. Other aspect to consider is the huge sensitivity of the molecular methods, which could even make sometimes difficult to interpret results; the detection of fungal DNA is not always indicative of mycotic infection or of the presence of an active invasive organism. The correct correlation of these methods with invasive disease, and the development of standardized and reproducible techniques constitute a critical goal within the molecular clinical microbiology area

A.- Chromosomal length polymorphisms. Karyotyping Electrophoresis.

The advent of electrophoretic techniques for separating the intact chromosomal DNA molecules of lower eukaryotes has provided during last two decades means of characterizing the chromosome sets of these organisms. These techniques have provided fundamental new information about the basic organization of the genomes of many species of fungi. These approaches are based on the electrophoretic separation of undigested genome DNA or in the comparison of genome macrorestriction patterns obtained by genome digestion with low frequency restriction endonucleases (Lopez-Ribot *et al*, 2000; Shin *et al*, 2004; Chen *et al*, 2005). Application of these protocols permits to obtain species or even strain specific profiles. Comparison of such profiles has been a great advance in the species differentiation within the genera *Candida*, *Saccharomyces*, *Kluyveromyces* and *Zygosaccharomyces*, in the study of anamorph-teleomorph relationships between *Candida*, *Kluyveromyces, Pichia* and *Saccharomyces* species, as well as for synonyms verification (Belloch *et al*, 1997).

B.- Mitochondrial DNA polymorphism (mtDNA).

Yeast are organisms showing a wide range of variability in the mitochondrial DNA size, ranging from 6 to 25 μm of length. In most yeast species, mitochondrial genome has circular topology. The use of mtDNA in yeast taxonomy has several advantages as: (a) small size, (b) high number of mitochondrial DNA molecules by cell, and (c) one single mitochondrial karyotype in each wild dikaryotic isolate (Belloch *et al*, 1977).
During long time, the main limitation of this technique was the difficult isolation of mithocondrial DNA. In 1990, Querol *et al* designed a rapid method to overcome this problem and later, in 2001, this new protocol was slightly modified by López *et al*, reaching an easy performed fast approach. The method permitts to analyse the mtDNA without previous isolation and purification requirements. The technique is based on the GC content differences between the nuclear DNA (nDNA) and the mtDNA, being the GC% around 40% in the former but ca. 20% in the later. This difference brings that when total fungic DNA is digested with restriction enzymes that only recognize GC rich regions, as for example *Msp*I, *Hae*III and *Cfo*I, all with 50% GC target site, the nDNA is overdigested giving rise to a high number of short fragments, that are not detected by conventional agarose gel electrophoresis. This characteristic permitts to assume that when total DNA digested with these endonucleases is subjected to agarose electrophoresis only the mtDNA fragments will be observed. These fragments will be ordered by size constituting species specific patterns (Fernandez-Espinar *et al*, 2000; Rycoyska *et al*, 2004) or even strain specific ones (Sabate *et al*, 1998).

C.- Restriction Fragments Length Polymorphisms (RFLP).

This technique is based on the the diferentiation between microorganisms by the comparison of the restriction patterns obtained by digestion of a chosen target DNA with restriction endonucleases. The degree of similarity of the generated patterns allows to establish correlations between species, whilst the existence of unique patterns permitts their use as identificative markers. This methods has been

succesfully used to differentiate between the species of genera *Candida*, *Cryptococcus*, etc. (Esteve-Zarzoso *et al*, 1999; Sabate *et al*, 2002; Deak *et al*, 2004; Pinto *et al*, 2004).

This technique has been succesfully applied using the ribosomal DNA region including the intergenic spacers ITS1 and ITS2, and the 5.8S rRNA enoding gene (Fig. 1) (Kurtzman 1994; Esteve-Zarzoso, 1999).

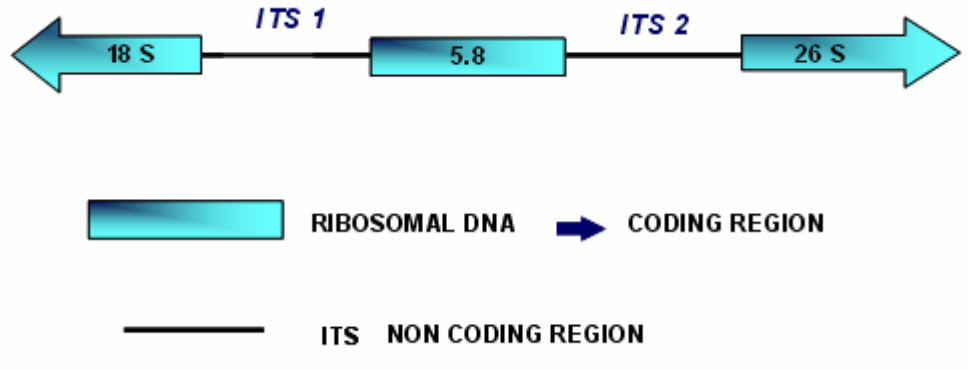

Fig. 1. Genomic organization of the 18S rDNA, ITS1, 5.8S rDNA, ITS2 and 26S rDNA.

The 5.8S gene has a highly conserved sequence showing a low intraespecific variability which is not enough to delimitate between coespecific strains. However, the ITS regions, which are non coding hypervariable ones, could permit depending on the case the identification at the intra or interespecific levels. In several studies, this technique is used in combination with PCR, what is called PCR-RFLP. In this combinative method, firstly specific DNA fragments are amplified by PCR and then, these amplicons are digested with restriction endonucleases to obtain specific patterns (Dendis *et al*, 2003; Llanos-Frutos *et al*, 2004).

D. Random Amplified Polymorphic DNA (RAPD)

This typing system is based on the PCR amplification of genomic DNA on the presence of a single short primer, often 10 nucleotides of length. Due to the use of a low annealing temperature (35-39 ºC), the primer binds to unespecific target sites, sites that are randomly distributed along the genome, what finally permits the obtention of DNA polymorphic amplicons (Fig.2). The amplified products are separated and visualized by gel electrophoresis. The use of RAPD permits to obtain the so called fingerprints which are combinations of different numbers of amplicons with different sizes, generating a pattern which is species or even strain specific (Orbera, 2004; Ergon and Gulay, 2005).

RAPDs have been used to develop genetic markers within several species and to discriminate between varieties of pathogenic yeasts. By means of this technique, the different *Candida* spp. serotypes have been distinghished (Alonso-Vargas *et al* 2000).

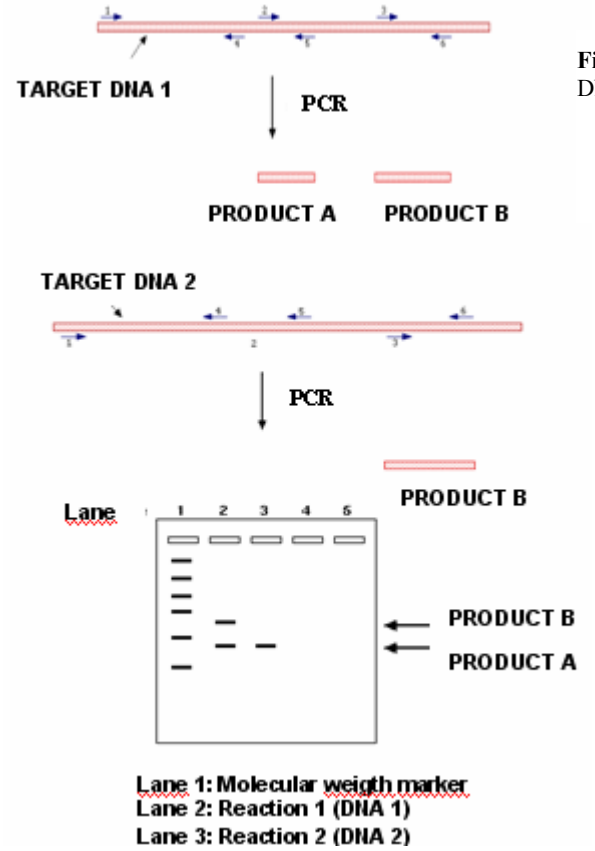

Fig.2. Random Amplified Polymorphic DNA (RAPD).

The number of fragments constituting the RAPD, the amplification intensity and the reproducibility of results depend on the amplification conditions, the components of the reaction mixture and the thermal cycler. These limitations make obvius the need of subjecting this technique to an optimization process, by which are defined the conditions for obtaining reproducible and trusthworthy patterns. However, although these restrictions could make RAPD to seem a very limited approach, it constitutes a highly discriminative typing technique yielding feasible results comparable within a laboratory. But, patterns should not be compared between different laboratories although conclusions can be correctly extrapolated.

Perurena *et al*, 2005, performed the genetic characterization of *C. albicans* strains recovered from the oral cavity of AIDS patients applying the PCR with 3 arbitrary primers (OPA-3, GACA 4 and M13); detecting different amplification patterns with each primer (Fig. 2).

E. Microsatellites analysis.

This molecular approach is based on the PCR amplification of fragments using oligonucleotides complementary to single repetitive sequences present in the target DNA. This repetitive sequences are called microsatellites. Some of the most frequently used are $(GTC)_5$, $(GTG)_5$, $(GACA)_4$, M13 phage (GAGGGTGGCGGTTCT). This technique differs of RAPD in the use of a higher annealing temperature (55ºC) in microsatellite analysis instead of 37ºC in RAPD. The application of a higher annealing temperature drives a more especific primer hybridization what consequently ensures a higher reproducibility (Botterel *et al*, 2001; Stephan *et al*, 2002; Dalle *et al*, 2003).

F. Short sequence repeats (SSRs) and variable numbers of tandem repeat (VNTR) loci

Short Sequence Sepeats (SSRs) are ubiquitous in eukaryotic genomes. Inter SSR (ISSR) fingerprinting is a typing technique developed such that no previous sequence knowledge is required for designing PCR primers. Primers based on a repeat sequence, such as $(CA)_n$, can be made with a degenerate 3'-anchor, such as $(CA)_8RG$ or $(AGC)_6TY$. The resultant PCR reaction amplifies the sequence between two SSRs, yielding a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping. PCR products are radiolabelled with 32P or 33P via end-labelling or PCR incorporation, and separated on a polyacrylamide sequencing gel prior to autoradiographic visualisation. A typical reaction yields 20-100 bands per lane depending on the species and primer. Several investigators have demonstrated that ISSR analysis usually detects a higher level of polymorphism than that detected with Restriction Fragment Length Polymorphism (RFLP) or Random Amplified Polymorphic DNA (RAPD) analyses.

Short sequence repeats (SSRs), potentially representing Variable Numbers of Tandem Repeat (VNTR) loci, were identified for the human-pathogenic yeast species *C. albicans* by computerized DNA sequence scanning. The individual SSR regions were investigated in different clinical isolates of *C. albicans*. Most of the *C. albicans* SSRs were identified as genuine VNTRs. They appeared to be present in multiple allelic variants and were demonstrated to be diverse in length among nonrelated strains. As such, these loci could provide adequate targets for the molecular typing of *C. albicans* strains. VNTRs encountered in other microbial species sometimes participate in regulation of gene expression and function as molecular switches at the transcriptional or translational level. Interestingly, the VNTRs identified in *C. albicans* often encode polyglutamine stretches and are frequently located within genes potentially involved in the regulation of transcription. DNA sequencing of these VNTRs demonstrated that the length variability was restricted to the CAA/CAG repeats encoding the polyglutamine stretches. For these reasons, paired *C. albicans* isolates of similar genotype, found as noninvasive colonizers or encountered in an invasive state in the same individual, were studied with respect to potentially invasion-related alterations in the VNTR profiles. However, none of the VNTRs analyzed thus far varied systematically with the transition from colonization to invasion (van Belkum A, 1999).

G. Amplified fragment length polymorphism (AFLP) analysis

Amplified fragment length polymorphism (AFLP) analysis has been shown to be a reliable method of reproducibly identifying medically important *Candida* species. Serial AFLP analysis of routine surveillance cultures for the identification and epidemiological examination of *Candida* sp. colonization has been succesfully assayed (Ball LM *et al*, 2004). These findings show that colonization with yeasts during transplantation is a complex and dynamic interaction between the host and the microorganism(s). AFLP analysis of surveillance cultures have been demonstrated to allow more accurate and informative epidemiological evaluations of pathogenic yeasts. Moreover, genotyping of *Candida* spp. clinical isolates by AFLP has revealed intraspecific genetic diversity among independent isolates and strain maintenance within patients.

In conclusion, the use of AFLP analysis as an identification method has shown very clear differences among medically important *Candida* species (Alcoba-Flórez *et al*, 1995). Furthermore, when screening a large collection of clinical isolates previously identified on CHROMagar as *C. albicans*, we found a misidentification rate of 6%. AFLP analysis is universally applicable, and the patterns can easily be stored in a general, accessible database. Therefore, AFLP might prove to be a reliable method for the identification of medically important *Candida* species.

H. Real Time PCR.

In the Real Time PCR, the amplification and detection proccesses occur simultaneously within the same closed vial, without the need of any ulterior action. Moreover, the use of fluorescence detection permits the quantification of newly synthesized DNA in each moment. The fluorescence emission during

the reaction is proportional to the amount of DNA produced. That allows to know and to monitor in each moment the kinetic of the amplification reactions (Higuchi *et al*, 1993).

The progress of the PCR reaction is measured by acquisition of data at regular intervals of time. The relative fluorescence units are graphically registered as a function of the number of cycles indicated in the abscises axis (McKillip and Drake, 2004). The fluorescence signal, which is proportional to the of amplified product, can be generated by an inespecific detection strategy, which is non dependent of the target sequence (fluorescent interbridge agents) or by a specific detection (hydrolysis probes or hybridazation probes).

The SYBR Green system (fluorescent interbridge) is based on the unspecific union of the fluorofore SYBR Green I to the minor groove of bicatenary DNA. SYBR Green I is fluorescent only when it is joined to DNA and this characteristic property is used to monitor the amplification process at the time the product is being generated. The confirmation of the sequence of the amplified product is performed by analysis of the fusion curve (Fig. 3).

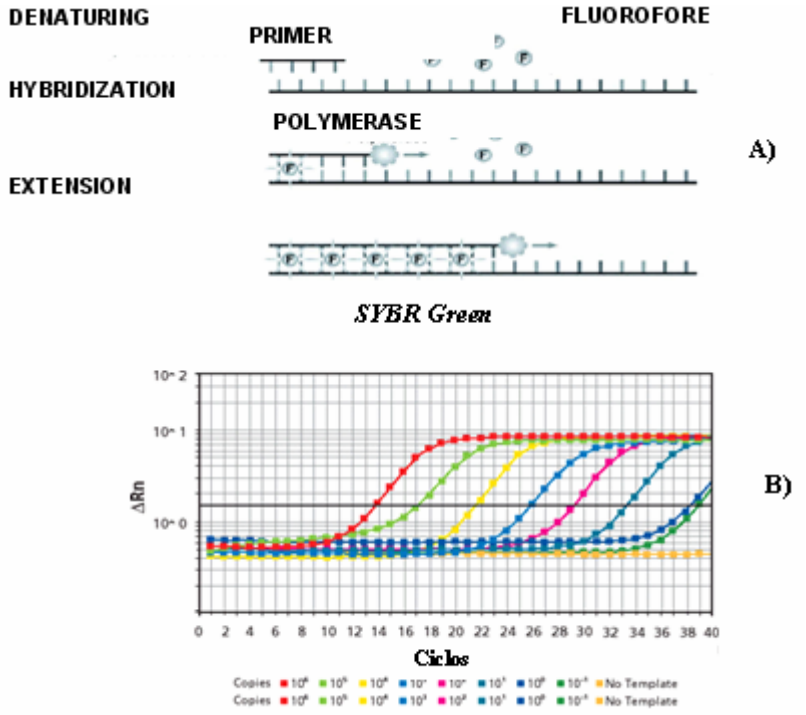

Fig. 3. Real time PCR. A) SYBR Green System. B) Quantification curve.

Once the PCR ends, the temperature of the termic chamber slowly increases and the fluorescence from each tube is measured. Since the bicatenary DNA starts to denature, the SYBR Green I fluorofore gets free what results in a fluorescence decay. Taking into account that each bicatenary DNA has its own fusion temperature, which depends on its GC content, the analysis of the fusion curve could be compared with the analysis in agarose gels of the size of the PCR product (Rijpens and Herman, 2002). This system is commercialized by Applied Biosystems (Foster City, California, EE.UU.) and by Roche Diagnostics (Mannheim, Germany) (Fig. 3).

The hybrolysis and the hybridization probes are based on the process of Fluorescence Resounding Energy Transfer (FRET) (Stryer, 1978), a process by which the energy of an excited donor fluorofore is transferred to an acceptor fluorofore, being the physical distance between both fluorofores of 70-100 Å. When donor and acceptor are tightly near, the fluorescence levels generated are very low or unexistent. But when the two fluorofores are separated, a fluorescence signal is emitted at a detectable wavelength.

In the hydrolysis probes, the 5´-3´nuclease activity of the Taq DNA-polymerase is used to digest the probe labeled in its 5´end with a fluorofore (reporter) and in the 3´ with a molecule which quenchs the fluorescence emitted by the fluorofore (Holland *et al*, 1991). The reporter fluorescence is mitigated by the quencher that is present in the same probe. Since the Taq DNA polymerase elongs the DNA chain from the primer, it moves and breaks the probe distancing then reporter and quencher (Fig. 4).

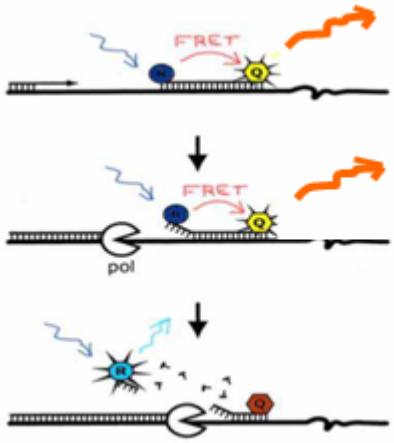

Fig. 4. Real time PCR. Hydrolysis probes (FRET: Fluorescent Resonance Energy Transfer).

As a result of the hydrolysis of the probe and the consequent separation of the two photochromes, the fluorescence intensity increases. The cycling PCR process results in an exponential amplification of the PCR product and the fluorescence intensity. This technique is known as TaqMan™ (Applied Biosystems).

On the other hand, the probes known as *molecular beacons*™ are oligonucleotides complementary to target DNA sequences within the gene studied (Tyagi and Kramer, 1996). These probes have a particular structure, showing a loop flanked by complementary inverted repeated sequences. They have a fluorofore in one end and a fluorescence quencher in the other, being both of them, fluorofore and quencher, covalently joined to the probe (Fig.5). When the probe shows the original looping structure, the quencher closed to the fluorofore does not permit fluorescence emitting. When the probe anneals with the target DNA, the loop structure disappears and the quencher is distant to the fluorofore. Then, the fluorescence is emitted and can be detected (McKillip and Drake, 2004) (Fig. 5).

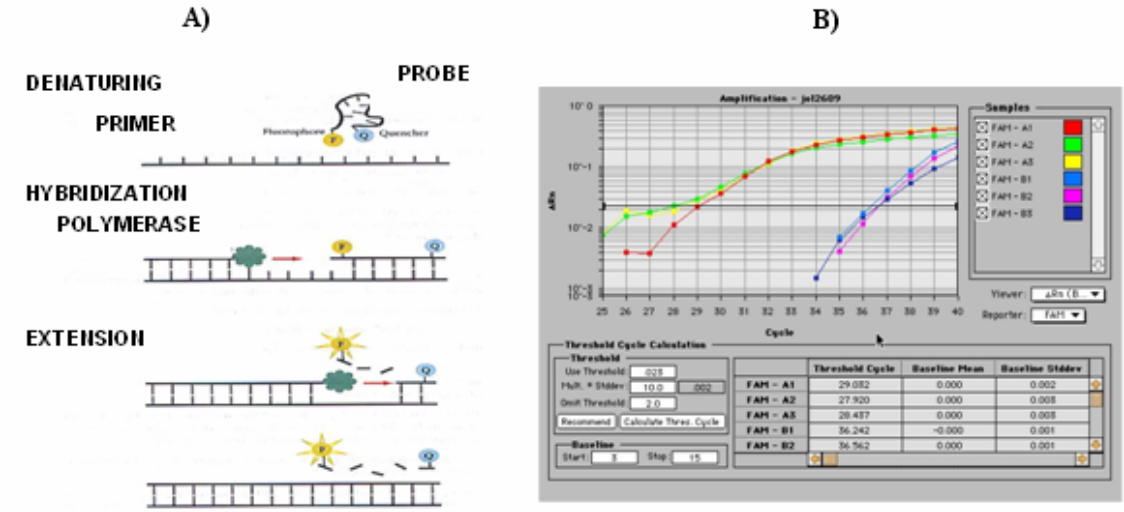

Fig. 5. Real time PCR. A) Taqman probes. B) Quantification curve.

In 2001, Guiver *et al* applied the *Taqman* system using species specific primers and probes for the identification of *C. albicans, C. glabrata, C. kefyr, C. krusei* and *C. parasilopsis*. The probes were labelled with three different fluorofores to permit the differentiation between species. In 2002, Selvarangan used the LightCycler method to distinguish between *C. albicans* and *C. dubliniensis*. Hsu also used in 2003 the same system to identify different *Candida* species. Five different species could be differentiated then, *C. albicans, C. glabrata, C. krusei* and *C. parasilopsis* and *C. guilliermondii.*

I. Sequencing of the amplified fragments.

The capacity to sequence nucleic acids has supposed a grat advance in Biology and Medicine. It has brought the possibility to know the primary structure of genes and to infer then the encoded function/s by comparison with well known sequences.
Several studies have used the direct sequencing of the ITS regions and the D1/D2 domains for yeast identification (Table 1).

Table 1. Studies focused on sequencing the ITS1/ITS2 regions and the D1/D2 domains.

Region	Reference	Genus or Species
ITS1/ITS2		
	Turenne, 1999	*Candida spp.* y *Saccharomyces. cerevisiae*
	Chen, 2000	*Candida spp., Cryptococcus spp., Rhodotorula spp.*
	Chang, 2001	*C. albicans, C. krusei, C. glabrata, C. guilliermondii, C. tropicalis, C. parasilopsis*
	Ahmad, 2002	*C. albicans, C. glabrata, C. parasilopsis, C. tropicalis*
	Selvarangan, 2003	*C. albicans, C. glabrata, C. parasilopsis, C. tropicalis, C. krusei , C. lusitaniae*
	Li, 2003	*Candida spp., Cryptococcus neoformans*
	Massonet, 2004	*Candida spp., Kluyveromyces spp.*
	Alcoba- Florez, 2005	*C. glabrata, C. nivariensis*
D1/D2		
	Kurtzman, 1997	*Candida spp., Picchia spp., Saccharomyces spp., Kluyveromyces spp.*
	Mannarelli, 1998	*Candida spp., Kluyveromyces spp.*
	Kurtzman, 2003	*Candida spp., Saccharomyces spp., Kuyveromyces spp.*

C.: *Candida*

J. Multilocus Sequence Typing (MLST)

Typing by the analysis of sequences of multiple loci is an approach based on the amplification and sequencing of inner fragments of housekeeping genes. In the case of yeast, different ribosomal and/or viurulence genes have been used with this porpouse (Vazquez and Berron, 2004). MLST detects variations ocurring in multiple loci by sequencing ca. 500 bp inner fragments of 7 different constitutive genes. For each gene, the different sequences detected within a species are assigned as different alleles and for each isolate the alleles in each of the 7 loci define its allelic profile or sequence type (ST) (Maiden *et al*, 1998). This typing procedure permits an exact assignation of the different isolates with the additional advantage that DNA sequences are not biased data easily interchanged by different laboratories and databases.

MLST was originally described for haploid organisms, but following a similar methodology has been used for typing *Candida*, which is diploid. In 2002, Bougnoux *et al* employed the method by sequencing inner fragments of 6 different housekeeping genes. In 2003, Tavanti *et al* obtained similar results using other housekeeping genes.

Conclusions and Perspectives

During last decades of the 20th Century, when the health concerns for the coming Century were predicted, diseases as cancer, AIDS or cardiovascular accidents were considered as the great problems to fight against. On the other hand, after the 50s, the therapeutic successes against infectious diseases by antibiotics administration made think that most infections would constitute a minor problem. However, the increasing appearence and dissemination of virulent infectious agents and resistance against most available available drugs to figth against them have made false that prediction. On the contrary, now in the 21st Century, as the World Health Organization pointed out already in 1999 (WHO/CDS/99.1), the infectious diseases are one of the most heavy charge for human populations.

In conclusion, as well as before 1950, human mankinds are in front of a situation in which the optimization of the fight against infectious diseases constitutes a priority. As part of this global objective, clinical microbiology needs a continuous optimization of the detection of infectious agents and their virulence and resistance genes at the specificity, sensitivity and velocity levels. This optimization permits to improve the prevention and treatment programs. Molecular typing techniques constitute a corner stone within this wide but necessary aim.

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