Infection, Genetics and Evolution 12 (2012) 1654–1660

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Genotyping of *Candida parapsilosis* from three neonatal intensive care units (NICUs) using a panel of five multilocus microsatellite markers: Broad genetic diversity and a cluster of related strains in one NICU

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ARTICLE INFO

Article history:
Received 29 February 2012
Received in revised form 22 June 2012
Accepted 23 June 2012
Available online 5 July 2012

Keywords:
Candida parapsilosis
Microsatellite
Molecular epidemiology
Neonatal intensive care unit

ABSTRACT

Candida parapsilosis (CP) ($n = 40$) isolated from an unselected patient population in the neonatal intensive care units (NICUs) of three US hospitals were collected over periods of 3.5–9 years. Two previously published microsatellite markers and three additional trinucleotide markers were used to produce multiplex genotypes, which revealed broad strain diversity among the NICU isolates with a combined index of discrimination (D) = 0.997. A cluster of eight related CP strains from four infants in a single NICU was observed. An extended collection of 24 CP isolates from the general population of that hospital showed that the cluster of NICU isolates was related to three isolates from general hospital patients. This microsatellite marker set is suitable to investigate clusters of colonizing and infecting strains of CP.

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1. Introduction

Candida parapsilosis (CP) is second to *Candida albicans* as the most common species causing invasive candidiasis or candidemia in pediatric patients whereas, in adults, *Candida glabrata* replaces CP (Zaoutis, 2010). CP can survive for weeks on inanimate surfaces and can cause candidemia and hematogenous dissemination (Traoré et al., 2002; Trofa et al., 2008). The yeast adheres to and forms biofilms on catheters and other indwelling plastic devices and, once embedded, is more resistant to azole antifungal agents (Tumbarello et al., 2007; van Asbeck et al., 2009a). The high glucose environment associated with parenteral nutrition contributes to biofilm formation. The property of CP as a skin colonizer facilitates transmission from an exogenous source, the hands of healthcare workers, to neonates during installation and maintenance of intravascular catheters (Yildirim et al., 2007).

For these reasons CP is notable for its involvement in hospital outbreaks (Clark et al., 2004) and as a cause of late onset sepsis (LOS) in the neonatal intensive care unit (NICU) (Fridkin et al., 2006; Kaufman, 2004; Stoll et al., 2002). Risk factors for CP candidemia in the NICU include parenteral nutrition catheters, prematurity, and very low birth weight (<1500 g). *Candida* species ranked third as a cause of LOS in pre-term infants in a major multi-center NICU study (Stoll et al., 2002) and as a significant cause of LOS among late preterm infants admitted to 248 NICUs in the United States between 1996 and 2007 (Cohen-Wolkowiez et al., 2009). The highest reported rate of candidemia in one European NICU (24%) was among infants with a gestational age of 23–28 weeks (Johnsson and Ewald, 2004).

Molecular epidemiology applied to infection control can reveal persistence and transmission of related strains in the hospital environment. Genotyping of CP has evolved from chromosomal electrokaryotyping (Carruba et al., 1991), arbitrarily primed PCR (Lehmann et al., 1992), and Southern blotting (Enger et al., 2001)

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1567-1348/$ - see front matter Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.meegid.2012.06.012

to the use of polymorphic microsatellite markers (PMM), (Lasker et al., 2006; Sabino et al., 2010). Successive methods improved in ease of use and discriminatory power. Efforts to apply multilocus sequence typing to CP isolates, however, were hindered because CP is diploid or aneuploid and possesses little (⩽0.1%) inter-strain DNA sequence variation (Fundyga et al., 2004; Odds and Jacobsen, 2008; van Asbeck et al., 2009b). Instead, the trend in CP genotyping is towards reliance on length variations associated with microsatellites (Brillowska-Dabrowska et al., 2009; Lasker et al., 2006; Sabino et al., 2010).

We revised a previously reported PMM genotyping scheme (Lasker et al., 2006) retaining two microsatellite markers and adding three trinucleotide repeat microsatellites. These markers were applied to a collection of 40 CP isolates from three NICUs in different US states, and a collection of CP isolates from the general population in two of the hospitals. The objectives were to: (i) refine an optimal set of microsatellites to aid in infection control measures since clusters of related CP strains have been reported to persist and circulate in the hospital environment (Reiss et al., 2008; Sarvikivi et al., 2005); (ii) detect potentially unrecognized clusters of related CP strains within a NICU; and (iii) compare the genotypic diversity of CP isolates among geographically separated hospitals.

2. Material and methods

2.1. Clinical isolates of C. parapsilosis and culture processing

The data set consisted of 40 CP isolates from three NICUs and, as an out-group, five CP isolates from an outbreak of CP candidemia among adults in a community hospital in Mississippi (Clark et al., 2004), (Table 1). Twelve isolates from Hospital A in Minneapolis, MN were obtained: 11 from infants in the NICU and one from an adult patient from the general hospital population, collected between August 1991 and January 2001. Sixteen isolates from Hospital B in Charlottesville, VA were obtained from 12 infants in the NICU between February 2003 and September 2006, and 24 additional isolates from the general patient population collected between 2006 and 2007. Five additional yeast isolates received from the Hospital B NICU were excluded by molecular identification; four were identified as *C. orthopsilosis* and one as *Candida metapsilosis* (Tavanti et al., 2005). Twelve CP isolates (11 from blood and one from CSF) from Hospital C in Philadelphia, PA were obtained from infants in the NICU between February 2003 and October 2007. An additional 13 isolates were collected between 2006 and 2007 from children in the general hospital C population (10 blood isolates, and one each from pleural fluid, a lung biopsy, and from tympanocentesis fluid).

Yeast isolates were identified by Hospitals A-C using morphologic and biochemical methods (Hazen and Howell, 2006). *C. metapsilosis* and *C. orthopsilosis* were identified by PCR amplification of the ITS region of rDNA with primers ITS1 and ITS4. The resulting sequences were submitted to the yeast data base of BiolomICS® (BioAware, Hannut, Belgium) accessed at the Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands) website, and were matched to either *C. metapsilosis* or *C. orthopsilosis* with ⩾99% certainty. Three of the *C. orthopsilosis* from this collection, and two *C. metapsilosis* isolates from CDC active surveillance for candidemia were screened for cross-hybridization against the PMM markers. For DNA extraction, cultures were grown at 30 °C on Sabouraud-glucose agar slants. Yeast cells were washed by centrifugation in deionized H_2O, resuspended in ATL solution (Qiagen Corp., Valencia, CA), and mechanically disrupted (5 min) with glass beads. The supernatant was centrifuged and Proteinase K (50 μg/ml in Tris–HCl buffer + 1 mM $CaCl_2$) was added and digested at 55 °C for 1 h. Heat-treated RNase A (50 μg/ml) was added and incubated for 15 min at 37 °C. Samples then were heated at 70 °C for 10 min and centrifuged. DNA was purified using the DNeasy Blood and Tissue Kit® (Qiagen) following the supplier's recommendations and quantitated using PicoGreen dye in a fluorescence analyzer (Molecular Devices, Sunnyvale, CA).

2.2. Identification of additional polymorphic loci

The CP genome database can be found at http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/. The FindPatterns® program (Accelrys, Genetics Computer Group, San Diego, CA) was used to screen CP strain ATCC 22019 for di- and tri-nucleotide repeats, and sequences with greater than eight repeat units were selected. To identify allelic variants, primer pairs were designed as close as possible to flank the microsatellite repeat region. Loci were screened by PCR according to Lasker et al. (2006) in eight randomly selected CP isolates and the amplification products were inspected for size polymorphisms after capillary electrophoresis. The 5′ ends of the forward primers were labeled with either 6-carboxyfluorescein or 7-hexachloro-6-carboxyfluorescein for analysis by capillary electrophoresis. Primer sequences, along with linkage group assignments and locations, are given in Table 2. The Blast algorithm at http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/ was used to identify chromosomal linkage groups and positions. Discriminatory power was calculated by the formula of Hunter and Gaston, 1988. The index of association (IA) was calculated using Multilocus 1.4 (Agapow and Burt, 2001).

2.3. Genotyping

Five microsatellite markers comprised the set used for genotyping. Of these, two, "B" and "G", were from the original set (Lasker et al., 2006) and three ("PB", "PC", "PD") were developed for this study. Subsequently we discovered that PB, PC, and PD amplified the same trinucleotide repeats as the CP4, CP1, CP6 (respectively) microsatellites reported by Sabino et al., 2010. The PMM genotype method applies only to CP *sensu stricto*. A sampling of the genetic species, *C. orthopsilosis* and *C. metapsilosis*, was tested for cross-hybridization. A total of 30 ng DNA was used per PCR reaction conducted according to Lasker et al. (2006) using the labeled primers described above. Following PCR, amplicons were sized by capillary electrophoresis on an ABI 3730 DNA Analyzer® (Applied Biosystems, Foster City, CA) coupled with GeneMapper® v. 4.0 software (Applied Biosystems). Alleles sizes were scored with respect to GeneScan™ 500 TAMRA™ Size Standard (Applied Biosystems) in the 35–500 nt range. The multiplex values of five PMMs were used to estimate pairwise genetic distances with the chord distance algorithm of Cavalli-Sforza and Edwards (1967) and the software program Powermarker version 3.25 (Liu and Muse, 2005). TREEview (Page, 1996) was used to construct dendrograms using the unweighted pair group method with arithmetic mean (UPGMA). To monitor for changes in microsatellite patterns over time, CP isolates V-1 and V-20 were passaged in vitro for 200 generations and re-tested against the microsatellite panel.

3. Results

3.1. Genome localization of the microsatellite markers and index of association

The original seven loci, A-G, were developed from a preliminary data set that included individual reads that had not been incorporated into larger contigs (Lasker et al., 2006). Since then, assemblies have been formed corresponding to the presumptive chromosomal linkage groups (Butler et al., 2009). From an analysis