

DNA Barcoding Analysis Confirmed Presence of Cell Wall Deficient Variants of Yeasts in Blood of Autistic Children and Their Mothers

Dilnora E. Gouliamova*, Roumen A. Dimitrov, Desislava B. Todorova, Nadya Markova

The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences

Abstract

Research on microbial pathogen's dormancy and survival strategy within the host is scarce. Yeast cell wall deficient variants (CWD) can persist *in vivo* for a long time and contribute to latent and chronic infections. In our study, we isolated yeast CWD variants from the blood of children with Autism Spectrum Disorder (ASD) and their mothers. The yeast CWD variants were converted into yeasts with the cell wall and were identified using DNA barcoding – sequence analysis of the variable D1-D2 domains of the nuclear large subunit (LSU nrDNA) and the internally transcribed spacer region ITS1+2 (ITS). All yeast strains were identified as *Candida parapsilosis*, and the strains were susceptible to six common antifungal agents.

Keywords: mycobiome, yeast pathogens

Резюме

Изследванията за стратегия на оцеляване на микробните патогени в гостоприемника са малобройни. Дрождеви варианти без клетъчна стена (БКС) са в състояние да персистират *in vivo* дълго време и могат допринасят за развитието на латентни и хронични инфекции. В нашето проучване дрождевите БКС варианти, изолирани от кръвта на деца с разстройство от аутистичния спектър (РАС) и техните майки бяха идентифицирани с помощта на ДНК баркодиране – анализ на последователността на вариабелните D1-D2 домейни на ядрената голяма рибозомна субединица (LSU nrDNA) и вътрешно транскрибируем спейсерен регион ITS1+2 (ITS). Всички изследвани щамове бяха идентифицирани като *Candida parapsilosis* и показаха чувствителност към шест противогъбични препарати.

Introduction.

Microorganisms such as bacteria, archaea, fungi, and viruses that inhabit the human body represent the microbiome. The microbiome plays a crucial role in human health (Cho and Blaser, 2012). Most microbes inhabiting the human body are commensal, and some are mutualistic. In healthy individuals as a part of the microbiome, fungi comprise the mycobiome and are found on the skin, in oral and nasal cavities, gastrointestinal tract, and uterus (Pammi *et al.*, 2013). However, in patients with acquired immunodeficiency syndrome (AIDS), cancer, organ transplantation, elderly and infants, commensal fungi can become pathogenic and cause severe infections.

Candida parapsilosis is a yeast species that are often isolated from blood cultures worldwide. (Pfalzer *et al.*, 2011). Moreover, some European, Asian, and South American hospitals leave behind *Candida albicans* (Trofa *et al.*, 2008). Also, *C. par-*

apsilosis is the most frequently isolated species in children's hospitals and is the leading cause of neonatal mortality (Piqueras *et al.*, 2021).

Until 2005 *C. parapsilosis* was separated into three groups. Genetic studies revealed that each group represents separate species: *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *Candida metapsilosis* (Tavanti *et al.*, 2005). The epidemiology, virulence traits, clinical manifestations, genetics, and antimicrobial susceptibility of *C. parapsilosis* have been intensively studied (Trofa *et al.*, 2008; Van Asbeck *et al.*, 2009). However, our knowledge is poor about this yeast's dormancy and survival strategy. The phenomenon of dormancy is one of the important biological properties of *Candida* species and is related to disease pathogenesis (Mattmann, 2001). It was shown that yeast CWD variants demonstrate pathogenicity as regular yeasts with cell walls. Infection of experimental animals with *Candida* yeast CWD variants has resulted in systemic candidiasis

* Corresponding author: dilnorag@gmail.com

(Kobayashi *et al.*, 1964; Mattmann, 2001).

Recent studies indicated that there is a link between fungal infections and central nervous system diseases, including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, schizophrenia, Rett syndrome, and multiple sclerosis (Alonso *et al.*, 2014; 2017; Severance *et al.*, 2017; Benito-León and Laurence, 2018; Strati *et al.*, 2018; Pisa *et al.*, 2020). There are a few studies on the association between fungi and neurodevelopment diseases such as ASD, which is the main interest of our research. ASD is characterized and diagnosed by poor social communication and unusual repetitive behaviours (Am. Psychiatr. Assoc., 2013). According to recent estimates, about one in 270 people globally has an ASD (Vos *et al.*, 2020). The etiology of ASD still is not clear. Studies indicate that both genetic and environmental factors contribute to the development of ASD (De Santis *et al.*, 2012; Kalkbrenner *et al.*, 2014; Geschwin and State, 2015; Willsey and State, 2015; Yang *et al.*, 2017). In our previous article in this field, we demonstrated that bacteria, filamentous fungi, and yeasts persist as CWD variants in children with ASD and their mothers (Markova, 2019). In this study, CWD variants of the filamentous fungi were converted to a typical detectable form and identified using serological tests and light microscopy. Converted bacterial and yeast forms were identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Recently MALDI-TOF MS analysis has become the method of the first choice for the identification of microorganisms due to its rapidity. However, the method has limitations in identifying cryptic and unknown yeast species. Therefore, in the present study identity of the yeasts that were isolated from the blood and urine samples of children with ASD and their mothers were confirmed using DNA barcoding analysis of sequences of LSU nrDNA and ITS region that are currently considered as the most reliable markers for the yeast identification (Kurtzman and Robnett 1998; Gouliamova *et al.*, 1998; 2016).

Materials and Methods

Blood samples

Three groups of people were studied: i) fifteen children (ages 3–12 years) diagnosed with ASD according to the standard international criteria; ii) group of their mothers (n=15; ages 30-47 years); iii) control healthy individuals (n=6; ages 12-22 years). Blood samples were aseptically collected from all investigated persons using K2E-EDTA

Vacutainer tubes (BD Vacutainer, Plymouth, UK). Informed consent for the use of the blood samples for research purposes was taken from mothers of children with ASD and healthy persons. All blood samples were handled and anonymized according to the national ethical and legal guidelines (Ethics Committee of Human Experimentation).

Isolation and transformation of yeast CWD variants into detectable forms

We used the previously described protocol to isolate CWD variants from blood samples (Markova *et al.*, 2016). Blood lysis was achieved with sterile distilled water at a strictly fixed v/v ratio and incubation of samples at room temperature for 30 min. The aliquots of lysed blood samples were inoculated in tubes with tryptic soy broth (TSB, Becton Dickinson) and incubated at 37°C for 72 hours. Then, strictly fixed aliquots from primary broths were further sub-cultured in two broth media TSB with gentamycin (100 µg/ml) and Sabouraud dextrose broth- SDB with chloramphenicol (50 µg/ml) and in parallel fixed aliquots were plated on two semisolid media - TSA with gentamycin of 100 µg/ml and SDA with chloramphenicol (50 µg/ml). The semisolid media solidified with 0.8% (w/v) agar (Fluca). SDB and SDA incubated at 25°C. Passages in broth and semisolid media were performed using the technique described in our previous study (Markova *et al.*, 2015). In control experiments, broths and semisolid media were inoculated with sterile saline, and the same technique did subsequent transfers. All cultures were periodically checked for the presence of yeast growth and morphological transformations within two months - direct light microscopic observations of native preparations and stained smears from broth and agar cultures done periodically.

DNA barcoding analysis

DNA extraction. Half of an inoculation loop of yeast culture was transferred into a microcentrifuge tube and stored at -20°C for at least 30 minutes. After incubation time, 500 µL lysis buffer solution (for 100 mL solution: 0.5 g SDS, 1.4 g NaCl, 0.73 g EDTA, 20 mL Tris-HCL (50 mM)) as well as 5 µL 2-Mercaptoethanol were added. The tubes were vortexed vigorously and incubated at 65°C for one hour. Samples were vortexed during incubation time at least once. Afterwards, DNA was purified with 500 µL phenol:chloroform: isoamyl alcohol (v:v 25:24:1). Tubes centrifuged at 12 000 rpm for 10 min. The supernatant was collected in a separate Eppendorf tube. DNA was precipitated with an

equal volume of isopropanol and then incubated at -20°C for 30 min. DNA was pelleted by centrifugation at 12 000 rpm for 10 min. DNA pellet was washed once with 200 µL of 70% aqueous solution of ethanol and centrifuged again at 12 000 rpm for 10 min. DNA pellet resuspended in 50 µL distilled water. The DNA concentration and purity were determined spectrophotometrically. DNA integrity is determined by 1% agarose DNA electrophoresis in TAE buffer (40 mM tris, 20 mM acetate, 1 mM EDTA, pH-8,3).

PCR amplification of LSU nrDNA domain and ITS1+2 region.

LSU nrDNA and ITS region were amplified using primer pair NL1/NL4 and V9D/Ls266 (Kurtzman and Robnett 1998; van den Ende and de Hoog, 1999). PCR products were sequenced using the same oligonucleotides used in the PCR using the BigDye kit (Applied Biosystems) by MacroGen (The Netherlands). Sequences were analyzed and edited using the Chromas program (<http://www.technelysium.com.au/chromas.html>). Nucleotide sequences of LSU nrDNA and ITS1+2 were compared with sequences from the GenBank database using the BLASTn search tool from NCBI (www.ncbi.nlm.nih.gov). All LSU rDNA and ITS sequences were deposited to the GenBank database.

LSU rDNA and ITS nucleotide sequences of all isolated strains showed 100% similarity. Therefore, LSU rDNA and ITS sequences of only one yeast strain 156 were deposited to the GenBank database under accession numbers OM220086/OM243925, correspondingly.

Susceptibility testing to antifungal agents

The assay was performed using Vitek-2 YST A17 card (Biomerieux, France) towards six common antifungal agents- fluconazole, voriconazole, flucytosine, micafungin, caspofungin, and amphotericin B.

Results

A list of isolated and identified yeast strains is presented in Table 1. Six strains 156, 158, 160,

162, 164, and 180 were isolated from the blood of children with ASD, and one yeast strain was isolated from the child's urine – 180-1. The three strains, 163, 165, 181, were isolated from the blood samples of mothers of autistic children. Control blood samples of six healthy individuals showed negative results on the presence of yeasts stains. BLAST analysis demonstrated that the nine strains showed 100% similarity in sequences of LSU nrDNA and ITS markers with *C. parapsilosis* CBS 604T (GenBank acc. number MH545914). Results of antifungal susceptibility testing showed that all isolated strains of *C. parapsilosi* were susceptible to six tested antifungal agents.

Discussion

Microbial CWD variants deserve special attention due to their persistence *in vivo* (Dominique, 1982; Mattmann, 2001). It was shown that, like bacteria, yeasts also can produce CWD L-bodies (Mattmann, 2001). Reversion of CWD L-bodies to typical yeast forms was observed in both *C. albicans* and *C. tropicalis* by Tunstall and Mattman (1961). *C. tropicalis* CWD variants were observed in patients with endocarditis (Rosner, 1966). Involution forms characterize persisting microbes, and the L-form of yeast is one of them.

Our previous article isolated and identified yeast CWD variants from the blood samples of six children, from the urine of one child and three mothers using MALDI-TOF MS (Markova, 2019). MALDI-TOF MS is done in three steps. First, analyzed particles are ionized and separated according to their mass-to-charge ratio. Second, the time necessary for the ions to reach a detector is determined. Third, obtained spectrum of mass to charge ratio and corresponding signal intensity is compared with a spectrum of known microorganisms from a database (Marklein *et al.*, 2009). The significant advantage of the MALDI-TOF MS identification method compared with DNA based ones is that it can be done in less than 10 minutes, and it does not require DNA extraction and PCR amplification

Table 1. List of yeasts strains isolated in this study

Strains		
Children/Blood samples	Children/Urine samples	Mother/Blood samples
A156 <i>C. parapsilosis</i>	-	-
A158 <i>C. parapsilosis</i>	-	-
A160 <i>C. parapsilosis</i>	-	-
A162 <i>C. parapsilosis</i>	-	M163 <i>C. parapsilosis</i>
A164 <i>C. parapsilosis</i>	-	M165 <i>C. parapsilosis</i>
A180 <i>C. parapsilosis</i>	U180 <i>C. parapsilosis</i>	M181 <i>C. parapsilosi</i>

steps. Nevertheless, the method has its limitations. First, it firmly depends on a database and respective analytical tools. As a consequence of this, there is no concordance between results obtained by platforms that use different databases. Second, it cannot discriminate reliably cryptic species and allocates them to a group, complex or genus level. Third, it is critical in MALDI-TOF MS analysis to include a sufficient number of strains for each fungal species grown under various growth conditions so that the database for the given species is robust enough to account for the species phenotypic variability. This is important for cryptic species of the *C. parapsilosis* complex that have limited protein sequence variability, and at the same time, they express a broad spectrum of phenotypes at different growth conditions (Tavanti *et al.*, 2010; De Carolis *et al.*, 2014). In the present study using sequence analysis of LSU nrDNA and ITS markers, we confirmed that six blood samples and one urine sample of children with ASD contained yeast *C. parapsilosis*. The analysis also confirmed that blood samples of three mothers of children with ASD also harboured *C. parapsilosis* yeasts.

Conclusion

DNA barcoding analysis confirmed that *C. parapsilosis* can persist in the blood of children with ASD and some of their mothers as CWD variants.

Acknowledgments

This study was supported by the grant KP-06-31/19 from Bulgarian National Science Fund.

References

- Alonso, R., D. Pisa, A. Rábano, L. Carrasco (2014). Alzheimer's disease and disseminated mycoses *Eur. J. Clin. Microbiol.* **33**: 1125-1132.
- Alonso, R., D. Pisa, A. M. Fernández-Fernández, A. Rábano, L. Carrasco (2017). Fungal infection in neural tissue of patients with amyotrophic lateral sclerosis. *Neurobiol. Dis.* **108**: 249-260.
- American Psychiatric Association (2013). Diagnostic and statistical manual of mental disorders (DSM-5). Arlington, VA: American Psychiatric Association Publishing.
- Benito-León, J., M. Laurence (2018). *Malassezia* in the central nervous system and multiple sclerosis. *Infection* **46**: 443-459.
- Cho, I., M. Blaser (2012). The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* **13**: 260-270.
- De Carolis, E., L. A. Lambert Hensgens, A. Vella, B. Posteraro, M. Sanguinetti, S. Senesi, A. Tavanti (2014). Identification and typing of the *Candida parapsilosis* complex: MALDI-TOF MS vs. AFLP. *Med. Mycol.* **52**: 123-130.
- de Santis, B., C. Brera, A. Mezzelani, S. Soricelli, F. Ciceri, G. Moretti, F. Debegnach, M. Bonaglia, L. Villa, M. Molteni, M. Raggi (2012). Role of mycotoxins in the pathobiology of autism: A first evidence. *Nutr. Neurosci.* **22**: 132-144.
- Domingue, G. J. (1982). Cell wall-deficient bacteria: basic principles and clinical significance. Reading, Addison-Wesley Publ London; pp. 312-322.
- Domingue, G. J. (2010). Demystifying pleomorphic forms in persistence and expression of disease: are they bacteria, and is peptidoglycan the solution? *Discov. Med.* **10**: 234-246.
- Geschwind, D. H., M. W. State (2015). Gene hunting in autism spectrum disorder: on the path to precision medicine. *Lancet Neurol.* **11**: 1109-1120.
- Gouliamova, D., R. Dimitrov, M. Smith, M. Groenewald, B. Guéorguiev, T. Boekhout (2016). DNA barcoding revealed *Nematodospora valgi* gen. nov., sp. nov. and *Candida cetoniae* sp. nov. in the *Lodderomyces* clade. *Fungal Biol.* **120**: 179-190.
- Kalkbrenner, A. E., R. J. Schmidt, A. C. Penlesky (2014). Environmental chemical exposures and autism spectrum disorders: a review of the epidemiological evidence. *Curr. Probl. Pediatr. Adolesc. Health Care* **44**: 277-318.
- Kobayashi, G., L. Friedman, J. Kofroth (1964). Some cytological and pathogenic properties of spheroplasts of *Candida albicans*. *J. Bacteriol.* **88**: 795-801.
- Mattmann, L. (2001). Cell wall deficient forms: Stealth pathogens 3th Edition, Boca Raton: CRC Press.
- Kurtzman, C. P., C. J. Robnett (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear, large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* **73**: 331-371.
- Marklein, G., M. Josten, U. Klanke, E. Müller, R. Horré, T. Maier, T. Wenzel, M. Kostrzewa, G. Bierbaum, A. Hoerauf, H. G. Sahl (2009). Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. *J. Clin. Microbiol.* **47**: 2912-2917.
- Markova, N., G. Slavchev, L. Michailova (2015). Presence of mycobacterial L-forms in human blood: Challenge of BCG vaccination. *Hum. Vaccin. Immunother.* **11**: 1192-1200.
- Markova, N., G. Slavchev, L. Djerov, A. Nikolov, T. Dimova (2016). Mycobacterial L-forms are found in cord blood: a potential vertical transmission of BCG from vaccinated mothers. *Hum. Vaccin. Immunother.* **12**: 2565-2571.
- Markova, N. (2019). Dysbiotic microbiota in autistic children and their mothers: persistence of fungal and bacterial wall-deficient L-form variants in blood. *Sci. Rep.* **9**: 13401.
- Pammi, M., Holland, L. Butler, G. Gacser, A. J. M. Bliss (2013). *Candida parapsilosis* a significant neonatal pathogen: a systematic review and meta-analysis. *Pediatr. Infect. Dis. J.* **32**: e206-16.
- Pfaller, M. A., G. J. Moet, S. A. Messer, R. N. Jones, M. Castanheira (2011). *Candida* bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008-2009. *Antimicrob. Agents Chemother.* **55**: 561-566.
- Piqueras, A., L. Ganapathi, J. Carpenter, T. Rubio, T. Sandora, K. Flett, J. Köhler (2021). Trends in pediatric candidemia: epidemiology, anti-fungal susceptibility, and patient characteristics in a children's hospital. *J. Fungi* **7**: 78.

- Pisa, D., R. Alonso, L. Carrasco (2020). Parkinson's disease: a comprehensive analysis of fungi and bacteria in brain tissue. *Int. J. Biol. Sci.* **16**: 1135-1152.
- Rosner, R. (1966). Isolation of *Candida* protoplasts from a case of *Candida* endocarditis. *J. Bacteriol.* **91**: 1320-1326.
- Severance, E. G., K. L. Gressitt, C. R. Stallings, E. Katsafanas, L. A. Schweinfurth, C. L. G. Savage, M. B. Adamos, K. M. Sweeney, A. E. Origoni, S. Khushalani, R. H. Dickerson Yolken (2017). Probiotic normalization of *Candida albicans* in schizophrenia: A randomized, placebo-controlled, longitudinal pilot study. *Brain Behav. Immun.* **62**: 41-45.
- Strati, F, Calabrò, A. Donati, C. De Felice, C. Hayek, J. Jousson, O. Leoncini, S. Renzi, D. Rizzetto, L. De Filippo, C. D. Cavalieri (2018). Intestinal *Candida parapsilosis* isolates from Rett syndrome subjects bear potential virulent traits and the capacity to persist within the host. *BMC Gastroenterol.* **18**: 57.
- Tavanti, A., L. A. Hensgens, S. Mogavero, L. Majoros, S. Senesi, M. Campa (2010). Genotypic and phenotypic properties of *Candida parapsilosis* sensu stricto strains isolated from different geographic regions and body sites. *BMC Microbiol.* **10**: 203.
- Trofa, D., D. Gácsér, J. Nosanchuk (2008). *Candida parapsilosis*, an emerging fungal pathogen. *Clin. Microbiol. Rev.* **21**: 606-625.
- Tunstall, L., L. Mattman (1961). L variation in *Candida* species. *Bacteriol. Proc.* pp. 83.
- Van Asbeck, E. C. K. V. Clemons, D. Stevens (2009). *Candida parapsilosis*: a review of its epidemiology, pathogenesis, clinical aspects, typing and antimicrobial susceptibility. *Crit. Rev. Microbiol.* **35**: 283-309.
- Gerrits van den Ende, A. H. G., G. S. de Hoog. (1999). Variability and molecular diagnostics of the neurotropic species *Cladophialophora bantiana*. *Stud. Mycol.* **43**: 151-162.
- Vos, T., S. S. Lim, C. Abbafati, *et al.*, (2020). Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study. *Lancet* **2396**: 1204-1222.
- White, T., T. Bruns, S. Lee, J. Taylor, M. Innis, D. Gelfand, J. Sninsky (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic. In: Innis, M. A., D. H. Gelfand, J. J. Sninsky, T. J. White (Eds.), *PCR Protocols: A Guide To Methods And Application*. Academic Press, San Diego, pp. 312-322.
- Willsey A. J., M. W. State (2015). Autism spectrum disorders: from genes to Neurobiology. *Curr. Opin. Neurobiol.* **30**: 92–99.
- Yang, C., W. Zhao, K. Deng, V. Zhou, X. Zhou, Y. Hou (2017). The association between air pollutants and autism spectrum disorders. *Environ Sci. Pollut. Res. Int.* **24**: 15949-15958.