International Journal of Contemporary Pediatrics
Thayikkannu AB et al. Int J Contemp Pediatr. 2023 Nov;10(11):1622-1628
http://www.ijpediatrics.com
pISSN 2349-3283 | eISSN 2349-3291

DOI: https://dx.doi.org/10.18203/2349-3291.ijcp20233228

Original Research Article

Characterization and scanning electron microscopy of *Malassezia* species isolated from central line tips of neonates on total parenteral nutrition

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Received: 31 July 2023
Revised: 18 September 2023
Accepted: 25 September 2023

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ABSTRACT

Background: The aim of this study was to demonstrate colonization by *Malassezia* species in the central lines of very low birth weight newborns on total parental nutrition (TPN). We also aimed to perform scanning electron microscopy (SEM) of central line tip cut sections to document the quality of the biofilm, cell structure, micro-colony characteristics, and the presence of extracellular matrix.
Methods: We collected central line tips of very low birth weight newborns (<1.5 kg) over a one-year period. We included a total of 63 samples, which were cultured on SDA slants with and without olive oil along with controls purchased from CBS Netherlands. We incubated the cultures at 32°C and observed them every two days for three weeks. Once growth occurred, we phenotypically identified the cultures and observed the central line tip cut sections with SEM.
Results: Among the 63 central line tips, two (3.1%) were colonized by *Malassezia*. We observed a visible biofilm on the tips. We confirmed the phenotypic identification of the isolates as *M. furfur* and *M. restricta* by gene sequencing.
Conclusions: Our study revealed *Malassezia c*olonization in the central line used for total parenteral nutrition. Hence, it is important to have a high index of suspicion towards *Malassezia c*atheter-related blood stream infection in newborns on TPN with a clinical picture of fever (in spite of antibiotic therapy), leukocytosis, thrombocytopenia with cardiac disease, and pulmonary infiltrates. The outbreak potential of *Malassezia w*arrants preventive steps, early identification and treatment.

Keywords: *Malassezia, M. furfur, M. restricta, CRBSI, TPN*

INTRODUCTION

Genus *Malassezia* consists of yeasts that colonizes the seborrheic parts of the body, often by utilizing the sebum, cerumen, and meconium as substrates.[1-6] These organisms are also seen in the lower gastrointestinal tract, as a part of the oral mycobiome, in the nasal vestibule as commensals, and have been recovered from the root canal and proven to

cause cavities. In newborns, *Malasseziae* are commonly seen in superficial lesions such as cradle cap, seborrheic dermatitis, and neonatal pustulosis.[7-12]

Neonates are colonized by *Malassezia* immediately after birth from either the mother or healthcare workers, where gestational age and duration of hospitalization are significantly associated with colonization. The overall colonization rate was found to be 41.5% to 75% in the first two weeks of admission. Many infants have *Malassezia* as an established cutaneous flora by 3-6 months. Positive skin cultures for *M. furfur, M. globosa, M. obtusa,* and *M. slooffiae* have been observed in neonates.[2-5,8]

Fungemia is known to occur post central line colonization. *Malassezia*, as an agent in catheter-related blood stream infection (CRBSI), has been documented since as early as 1979.[1] This "yeast-like fungus" is a known causal agent of potential outbreak in neonatal intensive care units (NICUs) among babies receiving TPN.[13,14] TPN comprises linoleic acid and oleic acid, both of which have carbon chains of n>12, which satisfies the lipophilic growth requirement of *Malassezia.* Therefore, neonates on TPN are vulnerable to colonization and infection.[15] Although catheter colonization commonly occurs secondary to skin colonization, *Malasseziae* can gain access during insertion of the catheter on the skin surface, through the hub or connecting port, through a contaminated infusate, or by hematogenous seeding. Such a colonization is asymptomatic and is not predictive of positive central line cultures or systemic illness.[1-8,16]

TPN facilitates the proliferation of *Malassezia* by providing the lipid source. These colonies can migrate and adhere to more than one site in long indwelling catheters by forming fibrin-platelet deposits outside and within the catheter lumen. They can also form biofilms to protect themselves from the host's immune defenses, and can be refractile to therapy. However, it is interesting to note that TPN is not necessary for the development of CRBSI. Though they form the normal flora, virulence factors such as high lipolytic activity, biofilm production, and absence of autoaggregation can prove *Malasseziae* as pathogens.[17,18]

This study was planned to look for and demonstrate colonization by *Malassezia* species on central lines of very low birth weight newborns on TPN. We also aimed to perform scanning electron microscopy (SEM) of central line tip cut sections to document the quality of the biofilm, cell structure, micro-colony characteristics, and the presence of extracellular matrix.

METHODS

We conducted this observational study in the Mycology Section of the Department of Microbiology and the Department of Neonatology SRIHER over a one-year period between September 2014 to October 2015 after obtaining approval from the institution's ethics committee (EC-NI/10/Oct/19/38). Over this one-year period, we collected the central line tips of very low birth weight newborns (<1.5 kg) who were receiving TPN. We included VLBW babies on TPN as study population. A total of 63 samples were collected, which were immediately cultured on Sabouraud's chloramphenicol agar (SCA) with an olive oil overlay and modified Dixons agar, and sent to the Mycology Section, where they were incubated at 32 °C and observed for three weeks. We used *M. globosa (CBS 7966), M. furfur (CBS 7019), M. restricta (CBS 7222), M. sympodialis (CBS 7877), and M. slooffiae (CBS 7956)* as controls, which we procured from The Central Bureau voor Schimmelcultures Fungal Biodiversity Centre, Institute of the Royal Netherlands Academy of Arts and Sciences (The Netherlands).

When we observed growth, we phenotypically identified it based on Gram staining, catalase tests, temperature tolerance, Tween assimilation pattern, esculin hydrolysis, and growth in the presence of Cremophor EL. We performed this phenotypic speciation according to the method described by Ashbee et al. The only known lipid-independent species is *M. pachydermatis*, which can grow in SDA without lipid supplementation. The only species that did not grow in modified Dixons Aagar (mDA) was *M. nana*. We performed the Gram staining according to the modified Hucker method. *Malassezia* showed broad based budding pattern.

We determined the production of the enzyme catalase by using the test tube method. First, we placed 2 ml of 3% H_2O_2 into a test tube. We then took colonies with the help of a sterile wooden applicator and dropped them into the test tube. We took brisk effervescence as a positive result, and no effervescence as a negative result. The only species with a negative result was *M. restricta.*

Temperature tolerance is an important adaptation to be observed, as it is species specific. *M. slooffiae* is thermos-tolerant and can grow at 45 °C. Certain species (e.g., *M. globosa* and *M. restricta)* grow beyond 32 °C, and some easily grow at 37 °C (e.g., *M. furfur and M. pachydermatis).*

We analyzed the ability of the yeast to utilize fatty acids and Tweens (i.e., Tween 20, 40, 60, and 80) according to Tween assimilation patterns formed using the pour plate culture method. We added 2 ml of overnight yeast suspensions (approximately 105 cfu/ml) to 16 ml of molten Sabouraud's dextrose agar at approximately 50 °C, which was left undisturbed until it solidified. We made four wells using a 2-mm punch, with one well in each quadrant. Each well was filled with 5 µl of individual Tweens and incubated at 32 °C for up to one week. We noted the degree of growth and precipitation patterns made by the yeasts around individual tweens in the wells. *M. obtusa, M. furfur, and M. slooffiae* do not precipitate tweens, whereas *M. globose and M. sympodialis* are precipitate-producing strains.

To exhibited β-glucosidase activity, we inoculated a loop full of fresh yeast into a tube of Tween 60 esculin agar and incubated it at 32 °C for five days. The splitting of esculin was revealed by the darkening of the medium. We used this test to distinguish species that utilized Tweens (i.e., *M. furfur, M. sympodialis, M. slooffiae, and M. cuniculi*) from other non-assimilating species.

We observed growth in the presence of Cremophor EL or PEG-35 castor oil only in *M. furfur*.[1] We used scanning electron microscopy (SEM) to image the cut tip sections and to document the quality and purity of the biofilm, cell morphology, budding pattern, structure, micro colony characteristics, and the presence of extracellular matrix.

In the SEM imaging of the colonies, we added a small amount of actively growing fresh culture of *Malassezia* to 1 ml of sterile distilled water and vortexed the mixture to make a uniform suspension. We spread 200 µl of this suspension on an adhesive carbon strip to fix it, which was allowed to air dry before being placed into an ion sputter chamber. Under high voltage, 24-carat gold formed ions and coated the yeasts on the prepared smear before were took SEM images. The SEM we used to be shown in Figure 1.

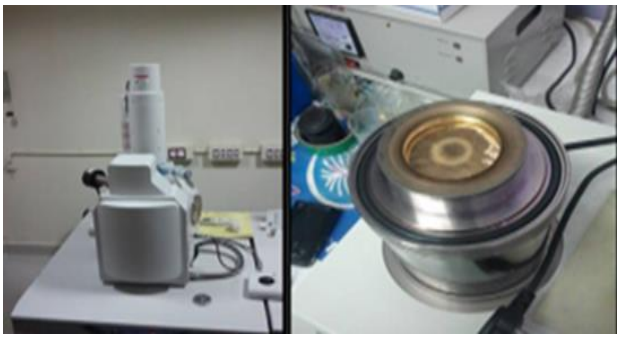

Figure 1: Scanning electron microscope.

RESULTS

Among the 63 central line tips, two (3.1%) were colonized by *Malassezia*, which we refer to as cases 1 and 2. We observed a visible biofilm on the tips. Phenotypically, we identified case 1 as M. furfur and case 2 as *M. restricta.* The SEM images of case 1 showed a well-formed biofilm occluding the entire lumen and extending around the tip. In further sections, the biofilm extended through the entire lumen. In the tip from case 2, we observed a visible semi-annular biofilm in the lumen and on the external surface. Figure 4 shows Phenotypic identification and biofilm of case 2.

We observed pure micro-colonies of yeast in abundant matrix in both tips. The surfaces of the yeast cells were smooth, and we observed various stages of unipolar broad based budding or blastic reproduction. We observed the classic collarette or bud scar where the daughter cells bud off from the mother cell.

On SEM, pure biofilms were observed. There were well organised micro colonies. Individual cells were smooth, had broad based budding, mostly unipolar, rarely bipolar and were suspended in an extracellular polysaccharide matrix.The biofilm seen in vivo and in vitro were similar (Figures 3 (2,9) and 4 (2,7)).

Confirmation of the species was done by gene sequencing at PGIMER.

The risk factors commonly seen are shown in Figure 2.

Case 1

An extremely preterm (24 weeks) male child born post IVF conception, as emergency lower segment caesarean (LSCS) in view of preterm onset of labor with preterm premature rupture of membranes (PPROM), was referred and admitted to the NICU for evaluation due to respiratory distress. The baby had extremely low birth weight, anemia of prematurity, and neonatal hyperbilirubinemia. On admission, the baby was intubated and later had multiple extubation failures, and was suspected to have chronic lung disease. Early steroid therapy was planned but withheld in view of sepsis. The baby had patent ductus arteriosus (PDA), which was treated with three doses of indomethacin. Surgical correction was deferred as the baby became asymptomatic. The baby was transfused with packed red blood cells on four different occasions to treat apnea and desaturation with anemia. On day 41, the baby developed septic shock, became hemodynamically unstable with an unrecordable blood pressure, and was started on inotropic support.

In view of the baby being extremely pre-term and having respiratory distress syndrome (RDS), amikacin and piperacillin-tazobactam were started after a septic work-up, which was escalated to imipenem on day four following apneic episodes. The baby's blood culture had grown *Acinetobacter baumannii.* Subsequent cultures showed growth of *Candida non-albicans*, which spread systemically causing renal fungal balls and ophthalmic involvement. Therefore, amphotericin B, which was initially started, was supplemented with voriconazole. The risk factors that we observed in the history are tabulated in Table 1.

A renal ultrasonogram showed left hydronephrosis secondary to fungal balls. There was no progression until day 41, when the baby developed acute kidney disease with decreased urine output. Renal function test showed hyperkalemia and elevated serum creatinine. Supportive care was initiated due to the baby's deteriorating renal parameters.

On day 44, the baby succumbed to fungal sepsis (Candidemia), shock, respiratory distress, acute renal failure, left hydronephrosis, PDA, extreme preterm, and low birth weight. Meanwhile, the tip of the central line was sent for culture. The tip had a visible biofilm, which was

cultured on SCA with an olive oil overlay and mDA. After four days, we noticed a creamy white smooth opaque growth. Gram staining showed the yeast *Malassezia*, which was speciated phenotypically as *M. restricta* and confirmed by gene sequencing at PGIMER Chandigarh. The tip sections were examined using SEM. Figure 3 shows the yeast *Malassezia*, which resembled *M. restricta*. In addition, well-formed pure biofilm was seen.

Case 2

A preterm (29 weeks+3 days) extremely low birth weight (780 g) baby boy was born by emergency LSCS to a primigravida with imminent eclampsia. At birth, the baby's cry color and activity was poor, his heart rate was less than 60 bpm, and he had poor respiratory effort. The baby was intubated due to lack of crying and developed hypotonia at birth.

On day two, the baby was suspected to have necrotizing enterocolitis, their ryles tube feed was stopped, and they were started on TPN. The baby had undergone surgery for meconium ileus. The baby tolerated RT feeds well postoperatively (ileostomy and stroma closure), and TPN was stopped. Post-septic work-up, amikacin, and piperacillin-tazobactam were stopped. Following *Acinetobacter baumannii* sepsis, meropenem was started and stopped on day 29. On day 38, the antibiotics vancomycin and imipenem were started, as the baby had become lethargic.

Table 1: Risk factors.

Risk factors	Case 1	Case 2
Extreme preterm and very low birth weight	Yes	Yes
Total parenteral nutrition	On TPN since day 2	On TPN since day 2
Steroid therapy	Yes	No
Long term antibiotics	Yes	Yes
Gram negative sepsis	Yes	Yes
Fungal infections	Candidemia, fungal sepsis	No
Isolate	*M. restricta*	*M. furfur*

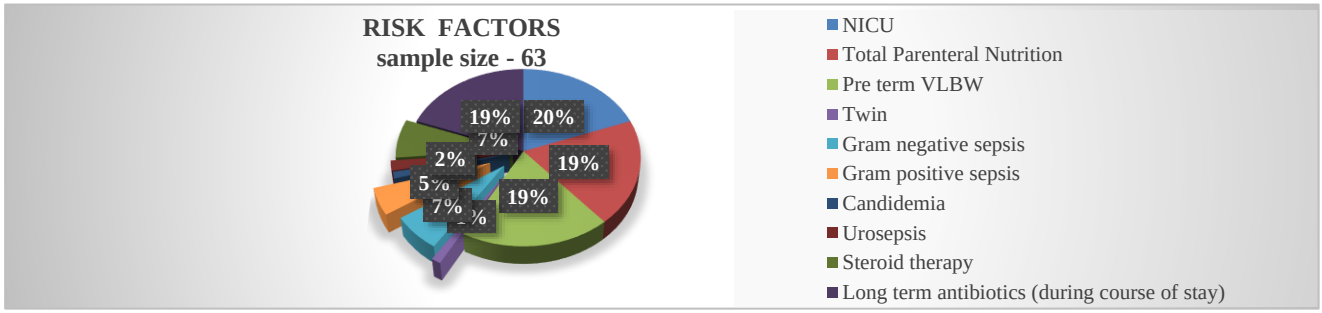

Figure 2: Risk factors in the study population.

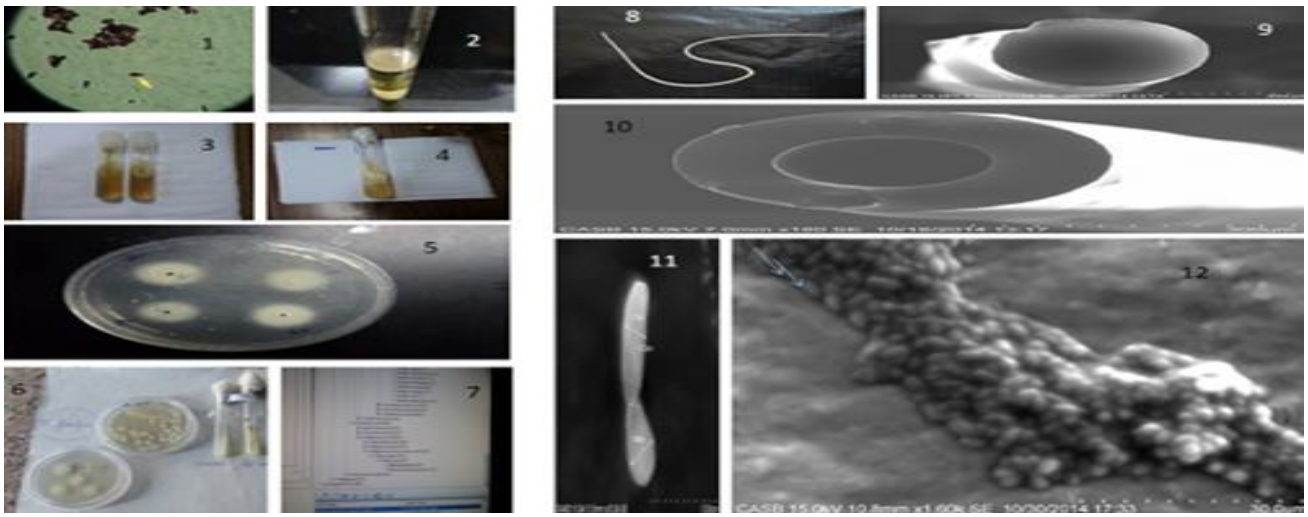

Figure 3: Phenotypic identification of *M. restricta* and scanning electron microscopy (SEM) images of case 1, 1- central line tip showing yellowish material/biofilm, 2- *Malassezia* in liquid media –semi annular surface growth, 3- Gram's stain showing the budding yeast, 4- tube catalase negativity, 5- SEM of the yeast *M. restricta*, 6- SEM of the tip showing biofilm on and inside the lumen, 7- SEM showing biofilm inside the lumen which is semi annular, 8- magnification of the Biofilm showing yeasts suspended in a matrix, 9- biofilm on the surface of the tip, 10- micro colonies, 11- magnified image of a yeast colony with visible matrix.

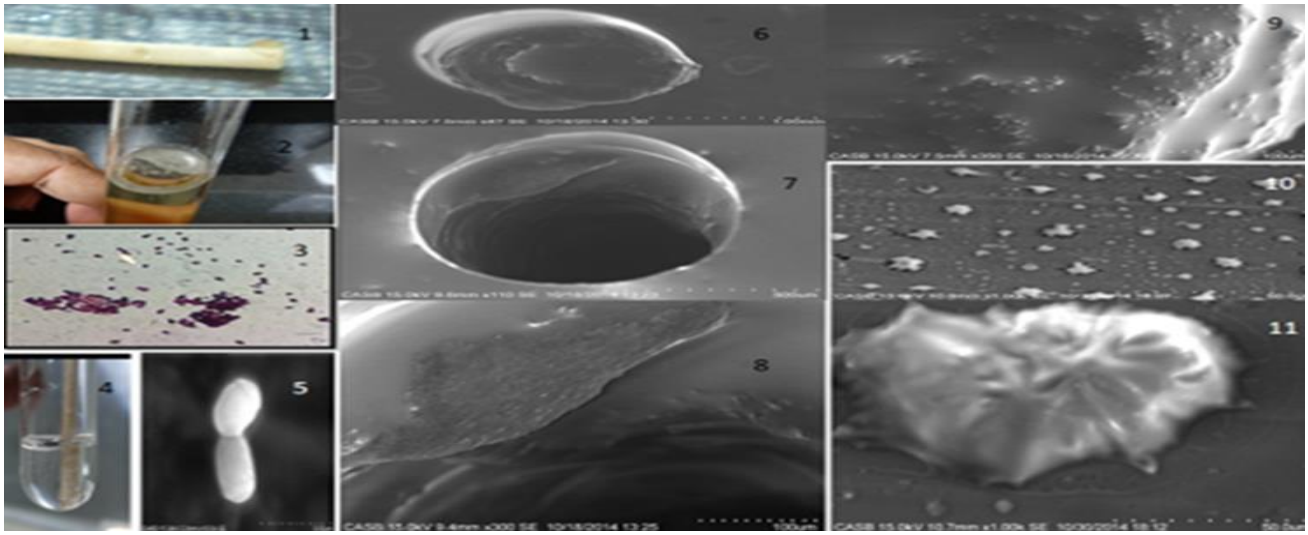

Figure 4: Phenotypic identification of *M. furfur* and scanning electron microscopy (SEM) imaging, 1- Gram's stain showing typical morphology of *M. furfur*, 2- broth culture showing complete surface growth at the soild liquid interface, 3- esculin not hydrolysed, 4- growth in the presence of Cremophor EL, 5- Tween assimilation test – Tweens 20, 40, 60 and 80 assimilated, 6- phenotypic identification, 7- MALDITOF MS database has *M. furfur*, 8- the central line tip showing completely occluded lumen, 9- SEM of the tip showing completely occluded lumen, 10- SEM of tip cut section showing biofilm all around the lumen, 11- yeast *M. furfur*, 12- microcolonies of *M. furfur* in an extracellular polysaccharide matrix.

DISCUSSION

The role of *Malassezia* as an agent in CRBSI is often overlooked and, therefore, underreported. In 1988, Azimi et al isolated *M. furfur* from 12 babies from two separate NICUs, five of which had evidence of sepsis.[13,14] *Malassezia* had caused occlusion of the infants' percutaneous central venous catheters, and visible growth was seen in clear catheters (TPN) connected to Silastic™ intravascular lines. Furthermore, culture positivity was observed, which agrees with the results of our study.

To the best of our knowledge, *M. restricta* as an agent in CRBSI has not been documented. At present, only *M. pachydermatis*, *M. furfur*, and *M. sympodialis* have been reported as agents in babies with CRBSI. As these yeasts were traced to the colonized hands of healthcare workers, they therefore have outbreak potential, as reported at the Louisiana State University Medical Center NICU. The non-lipid dependent *M. pachydermatis* has been isolated from blood cultures, tip cultures, urine, eye discharge, tracheal aspirates, and cerebrospinal fluid, proving its dissemination. According to Larocco et al, *M. pachydermatis* can persist on medical machines, and it can be resistant to normal disinfection processes.[16] *M. sympodialis* and *M. furfur* have been isolated as colonizers from adult intravascular catheters tips. Curvale-Fauchet et al and Kessler et al reported peripheral thromboembolism caused by the same agent.[17-20]

M. restricta

M. restricta is a solely human pathogen that is seen among normal microbiota on the scalp, both in health and in disease. It causes tinea versicolor, seborrheic dermatitis, and also aggravates atopic dermatitis. *M. restricta* causes culture-negative non-documented infective endocarditis. As *M. restricta* cross-reacts in indirect immunofluorescent assays, it can possibly be wrongly identified as Candida. However, unlike *Candida*, *M. restricta* is intrinsically resistant to echinocandins.[18,19]

M. restricta in disease is not just under-identified but also underreported. However, *Malassezia spp*. are now increasingly reported among premature babies on TPN.[18] *M. restricta* is also said to exacerbate irritable bowel disease, acute appendicitis, and colitis in mouse models. It is also implicated in endophthalmitis keratitis and seborrheic blepharitis.[13,21-23]

Mularoni et al reported *M. restricta* pneumonia in solid organ transplant recipients, which was diagnosed by pathological stains (e.g., Gomori's Methenamine silver stain, Periodic Acid Schiff stain), histology, molecular methods (e.g., polymerase chain reaction), and 18S-sequencing. The patients recovered well with antifungal therapy consisting of itraconazole, voriconazole, or amphotericin B.[20] While studying aerosols in hospital settings, Habibi et al observed 0.74% of the total to be *M. restricta*, making aerosols a probable route of infection.[21]

Suzuki et al described the difficulties in the lab diagnosis of *M. restricta* in mycotic keratitis, as it is unusual and also culture-negative when a lipid is not supplemented. They used microscopy, PCR, and DNA typing of ITS2 and 5.8S ribosomal DNA to identify *M. restricta* with 99% homology. Their treatment strategy comprised antifungals, oral itraconazole 150 mg/day, topical 5% pimaricin

ointment, and topical 0.2% miconazole, and the patient recovered in five weeks. There are case reports of keratomycosis in humans and dogs, where *M. restricta* and *M. furfur* were isolated from humans and *M. pachydermatis* from dogs. *M. furfur* was identified in corneal samples from a case of infectious crystalline keratopathy and blepharitis in a patient receiving long-term topical corticosteroid therapy. *M. restricta* is also documented to have caused ulcerative keratitis following soil contamination.[22]

Zareei et al assessed *Malassezia* biofilm formation and concluded that *M. restricta* was more capable of forming a biofilm than *M. globosa*, and, over time, the *Malassezia* biofilms had matured. This ability to form biofilms makes this pathogen one to look out for, as it can cause life-threatening fungemia.[24,25] In 2017, Angiolella et al observed that *Malasseziae* are hydrophobic and produce biofilms by actively producing extracellular matrix on abiotic surfaces.[26] In SEM studies, they observed abundant extracellular matrix, as in our case after 48 hours. 63% of their strains had high biofilm production, as well as medium to high hydrophobicity and/or adherence. They were able to demonstrate a correlation between biofilm formation, hydrophobicity, and adherence in approximately 60% of the isolates. They concluded that these virulence factors contributed to *Malasseziae* changing from being a commensal to being a pathogen, and in 2018, they also concluded the same for *M. furfur*.[24,26]

Complications of CRBSI

Complications of CRBSI include peripheral thrombophlebitis, thromboembolism, catheter occlusion, adhesion of the catheter to the endothelium of the vein, and endocarditis with intracardiac mass.[17-19] CRBSI treatment is easy and the prognosis is good when started early. Stopping TPN and removal of the colonized indwelling catheter followed by intravenous liposomal amphotericin B shows good therapeutic results. Weiss et al. have documented a neonate that recovered from *Malassezia* sepsis on stopping TPN without catheter removal, removing the requirement of lipophilic growth.[27-30]

Limitations

This study will be valuable if done with larger sample size and over a longer study duration in this study population. A correlation between colonization / infection is difficult in a newborn without regional/centre data. A detailed study including all those at risk in comparison to healthy individuals correlating with their cutaneous commensal flora will give valuable details on pathogenesis and increase our understanding of biofilm associated hospital acquired infections.

CONCLUSION

Clinical signs of fever despite antibiotic therapy, leukocytosis, thrombocytopenia with cardiac disease, and pulmonary infiltrates in babies on TPN should raise clinical suspicion for *Malassezia* fungemia following colonization. It is important to note that even in fulminant fungemia, 1,3-β-D-glucan is not elevated. All 18 members of this genus are not similar in virulence. However, their outbreak potential warrants preventive steps, periodic NICU surveillance, infection control measures, and early identification and treatment.

We cannot comment on the pathogenicity in either case, as blood culture in both case 1 and case 2 was done using BACTEC blood/fungal culture broth without lipid supplementation, incubated for five days, and was culture negative. However, if additional lipid supplements had been added, *Malassezia* could have grown because the TPN line tip was colonized, as proven by culture and SEM. Early diagnosis can be accomplished by matrix assisted laser desorption ionization time of flight mass spectroscopy (MALDITOF MS), nested PCR, quantitative real-time PCR, which can detect even low concentrations, biotyping using enzymatic methods, pulsed-field gel electrophoresis, random amplification of polymorphic DNA, DNA sequence analysis, restriction analysis of polymerase chain reaction amplicon of ribosomal sequences, amplified fragment length polymorphism, denaturing gradient gel electrophoresis, and terminal fragment length polymorphism.

ACKNOWLEDGEMENTS

Authors would like to thank Prof. P. Palani, CAS Botany, University of Madras for his guidance and support in performing SEM studies, and are grateful to Prof. Dr. Arunaloke Chakrabarthi Former Head, Microbiology, PGIMER for kindly confirming the isolates.

Funding: Indian council of Medical Research, New Delhi India, under the TSS Fellowship
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Thayikkannu AB, Kindo AJ, Veeraraghavan M, Panchanathan S, Prakash A, Ninan B. Characterization and scanning electron microscopy of Malassezia species isolated from central line tips of neonates on total parenteral nutrition. Int J Contemp Pediatr 2023;10:1622-8.