Outbreak of *Pichia kudriavzevii* fungaemia in a neonatal intensive care unit

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Abstract

**Purpose.** Fungaemia is associated with substantial morbidity and mortality in neonates admitted to neonatal intensive care units (NICUs). We report an outbreak of fungaemia in a NICU due to rare yeast, *Pichia kudriavzevii* (a teleomorph of *Candida krusei*). To the best of our knowledge, this is the first report of neonatal sepsis due to *P. kudriavzevii*.

**Methodology.** Between August and September 2014, blood cultures from nine neonates diagnosed with late-onset sepsis in the NICU yielded yeast-like organisms. The molecular identification and typing of these isolates was performed by sequencing the D1/D2 region of 26S rDNA and fluorescent amplified fragment length polymorphism (FAFLP) respectively. Antifungal susceptibility was tested by broth microdilution as per the Clinical Laboratory Standards Institute (CLSI) guidelines. Sampling from environmental sources and the hands of healthcare workers (HCWs) in the NICU was performed.

**Results.** Of the nine neonates, eight were preterm and six had very low birth weight (VLBW). Thrombocytopenia was present in two neonates. Sequencing identified all the isolates as *P. kudriavzevii* and FAFLP showed their clonal origin. Antifungal susceptibility testing revealed the susceptibility of all isolates to the antifungals tested. Treatment with voriconazole was advised. However, only seven neonates were treated successfully and discharged after improvement, whereas two were lost for follow-up. Cultures from the environment and the hands of HCWs were negative. The outbreak was controlled by the strict implementation of infection control practices.

**Conclusion.** This study emphasizes the importance of accurate identification of the aetiological agent of sepsis and vigilant monitoring for the possibility of an outbreak in NICUs.

INTRODUCTION

Nosocomial sepsis, also known as late-onset sepsis or healthcare-associated sepsis, is a very common complication in neonates hospitalized in the neonatal intensive care unit (NICU) [1, 2]. Fungi are the third most common cause of late-onset sepsis [3]. Fungal sepsis is associated with substantial morbidity and mortality [2, 4].

Risk factors for the development of fugal sepsis in neonates include intrinsic factors such as the immaturity of the immune system and increased permeability of the skin/mucous membrane, and extrinsic factors such as central vascular access, parenteral nutrition, broad-spectrum antibiotics, postnatal steroids, mechanical ventilation, etc [1, 4]. Environmental factors such as place of delivery, flora of delivery room/NICU and infection control practices also contribute to the incidence of sepsis in neonates [2].

*Candida* species are the commonest cause of late-onset fungal sepsis known to be transmitted horizontally, resulting in outbreaks [5–9]. Outbreaks of fungal sepsis due to common *Candida* species are well documented in the literature. Of late, reports associating nosocomial fungal sepsis with unusual *Candida* species have also been published [3]. However, very few studies have demonstrated clusters of fungal sepsis due to teleomorphic forms of non-*albicans* species identified using molecular DNA-based sequencing and typing methods [5].

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**Keywords:** Pichia kudriavzevii; fungaemia; NICU; antifungal susceptibility.

**Abbreviations:** CLSI, Clinical Laboratory Standards Institute; CPAP, Continuous Positive Airway Pressure; FAFLP, Fluorescent Amplified Fragment Length Polymorphism; HCW, Health Care Workers; HD NICU, high dependency NICU; LBW, Low Birth Weight; NCCPF, National Culture Collection of Pathogenic Fungi; NGT, Naso Gastric Tube; NICU, Neonatal Intensive Care Unit; PPE, Personal Protective Equipment; SDA, Sabouraud’s Dextrose Agar; UPGMA, Unweighted Pair Group Method with Arithmetic mean; VLBW, Very Low Birth Weight.
The manifestations of fungal sepsis are similar to those of bacterial sepsis and are often nonspecific. Timely and accurate laboratory diagnosis of fungal sepsis is thus very important, not only for management of the patient, but also for the control of outbreak. Although blood culture and conventional mycological techniques are routinely used for laboratory diagnosis, they may not always provide time-critical results. Molecular methods, in addition to providing timely results, are useful for typing and confirmation of strains in an outbreak.

We report an outbreak of fungal sepsis in a tertiary care NICU due to Pichia kudriavzevii (a teleomorph of Candida krusei). Fluorescent amplified fragment length polymorphism (FAFLP) showed that they were all clonal in origin.

**METHODS**

**Case definition**

Cases of late-onset fungal sepsis was defined as neonates with at least one positive blood culture 72 h after birth.

Between 25 August and 12 September 2014, nine neonates admitted to the NICU of the tertiary care hospital attached to Bangalore Medical College and Research Institute, Bangalore were diagnosed with late-onset sepsis. The nine neonates were admitted to different parts of the NICU – the outborn NICU, the inborn NICU and the high-dependency (HD) NICU.

**Microbiological investigation**

One set of blood samples was drawn from the peripheral vein of each of the nine neonates using aseptic techniques. The blood cultures of all the nine neonates yielded growth of light cream-coloured colonies. Subcultures on Sabouraud’s dextrose agar (SDA) yielded growth of butyrous and light-cream-coloured colonies after 24 h of incubation (Fig. 1). We collected clinical data for all nine neonates retrospectively by reviewing patients’ clinical charts.

**Environmental surveillance**

Environmental samples were obtained from different parts of the NICU – from patients’ bedding, incubators, sinks, the tops of trolleys, respiratory-care equipment, catheter sites, intravenous fluids, etc [10]. We also collected 28 hand-imprint samples from HCWs in the NICU [11]. The culture from the hand imprint of one nursing staff member grew yeast, while the other environmental samples were negative for fungal growth.

**Molecular investigation**

All the nine blood culture isolates and one culture isolate from the hands of a nursing staff member in the NICU were referred to the National Culture Collection of Pathogenic Fungi (NCCPF), Postgraduate Institute of Medical Education and Research, Chandigarh for identification, molecular typing and antifungal susceptibility testing.

**Genomic DNA extraction**

Genomic DNA extraction from the yeast was carried out from 24 h colonies on Sabouraud’s dextrose agar. The cells were subjected to bead beating in the presence of lysis buffer [400 mM Tris-HCl (pH 8.0), 60 mM ethylene diamine tetra acetic acid (EDTA) (pH 8.0), 150 mM NaCl, 1 % sodium dodecyl sulfate] and extracted using the phenol/chloroform/isooamyl alcohol method. The DNA in the aqueous phase was precipitated by the addition of ice-cold ethanol, pelleted, washed once with 70 % ethanol, air-dried and resuspended in TE buffer (10 mM Tris/HC, 1 mM EDTA, pH 8.0). The DNA was stored at –20 °C until use.

**Amplification and sequencing of the D1/D2 region of the 26S ribosomal RNA gene cluster**

The amplification and sequencing of the D1/D2 region of the 26S rDNA were performed with the primer pairs NL-1 (5’-GCATATCAAAGCAGGAGGAAG-3’) and NL-4 (5’-GGTCGGGTTCCTATACAGCGG-3’) [12] in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The PCR products were subjected to a sequencing PCR with the above-mentioned primers using the Big Dye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were analysed on an ABI 3130 genetic analyzer (Applied Biosystems) using an in-house facility. The sequences were checked for their identity in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

**Fluorescent amplified fragment length polymorphism (FAFLP)**

Molecular typing of the isolates was performed using fluorescent amplified fragment length polymorphism technique as described earlier with some modifications [13]. EcoRI and HindIII restriction enzymes (New England Biolabs, Ipswich, MA, USA) and corresponding adapters were used. Amplification was performed using pre-selective primers of EcoRI (5’-GACTGGGTATCCAATTCTC-3’) and HindIII (5’-GACTGGGTACCCAGCTT-3’). The HindIII primer with one selective residue (5’-GACTGGGTACCCAGCTTT-3’)

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**Fig. 1.** 24 h colonies of yeast on Sabouraud’s dextrose agar plate after subculture.
and the EcoRI (6-FAM-labelled) primer with two selective residues were used (5’-GACTGCGTACCAATTCAC-3’). Capillary electrophoresis of the amplified products (labelled with 6-carboxyfluorescein) and LIZ 500 (standard marker) was performed in an ABI 3130 genetic analyzer. Typing data were imported into BioNumerics version 6.6 software (Applied Maths, Ghent, Belgium). A phylogenetic tree was constructed by the unweighted pair group method with arithmetic mean (UPGMA) with 1000 bootstrap replications in combination with Pearson’s correlation coefficient. Candida albicans was used as an outgroup for analysis.

Antifungal susceptibility testing

The antifungal susceptibility of the isolates was tested in respect of amphotericin B, fluconazole, voriconazole, itraconazole, posaconazole, caspofungin, anidulafungin and micafungin using the broth microdilution method as per the M27-A3 guidelines of the Clinical Laboratory Standards Institute (CLSI) [14]. As P. kudriavzevii-specific breakpoints are not available, the antifungal susceptibility pattern was interpreted in the manner recommended for Candida species in the CLSI M27-S3 guidelines.

RESULTS

Nine cases of late-onset fungal sepsis were documented in the NICU during the study period. No other cases of fungal sepsis were detected immediately prior to the described outbreak. The first case of late-onset fungal sepsis was observed in a 4-day-old preterm neonate with very low birth weight (Table 1), who had been admitted to the inborn NICU for preterm care. The patient developed symptoms of sepsis and icterus on day 3 after admission and complete blood counts revealed thrombocytopenia for which platelet transfusion was initiated. A blood sample was sent for culture and treatment with antibiotics was initiated (amikacin – 15 mg kg⁻¹ i.v. OD for 7 days and cefotaxime 50 mg kg⁻¹ iv BD for 7 days). The blood culture grew yeast. Antifungal therapy could not be initiated as the patient was discharged against medical advice and lost to follow-up.

The second neonate was a 4-day-old preterm neonate. The time lag between the detection for the first and second patients was 2 days. The third case was a term neonate born to a hepatitis B-positive mother, and was admitted to the NICU with a diagnosis of late-onset sepsis. After the third case was identified 4 days after the first case, an outbreak of fungal sepsis was suspected and an epidemiological investigation was initiated immediately. In view of the outbreak, the importance of strict hand hygiene was reinforced to all medical staff.

In total, nine neonates were diagnosed with late-onset fungal sepsis over a span of 2.5 weeks. (Table 1). The age at the onset of fungaemia ranged from 4 to 28 days. Thrombocytopenia (platelet counts <100 000 mm⁻³) was the early laboratory finding in two cases. The mean gestational age was 32.4 weeks and the mean birth weight was 1.6 kg. The risk factors included preterm birth, low/very low birth weight,
neonatal hyperbilirubinemia, prior or present admission to a NICU, continuous positive airway pressure (CPAP), a naso-gastric tube (NGT), intubation/ventilator and being a triplet.

For all nine neonates, treatment with voriconazole (4 mg/kg/day) was advised. However, two patients went against medical advice and hence could not be followed up. The other seven babies who were treated with voriconazole improved and were discharged.

Environmental investigation
The surveillance cultures from the inanimate environment were all negative for yeasts or moulds. Of the 28 hand-imprint cultures taken from HCW, yeast was isolated from the hand of one member of the nursing staff. This yeast was identified as *Wickerhamomyces anomalus*.

Molecular identification and typing
The nine isolates from the blood samples from the nine neonates were identified as *P. kudriavzevii* by sequencing of the D1/D2 domain of the large subunit ribosomal DNA. Genetic relatedness among the clinical isolates was analysed by FAFLP assay (Fig. 2). FAFLP showed that all nine isolates were clonal in origin/identical to each other. The isolates grouped in two clusters – a major cluster of eight isolates with high bootstrap values (96.6–99.3 % similarity) and a minor cluster that contained a single isolate with 76.6 % similarity with the major cluster.

The source of the outbreak could not be traced, in spite of thorough environmental investigation.

Antifungal susceptibility testing
*In vitro* susceptibility testing revealed the susceptibility of all the isolates to amphotericin B (1 µg ml⁻¹), voriconazole (0.25 µg ml⁻¹), itraconazole (0.25 µg ml⁻¹), posaconazole (0.5 µg ml⁻¹), caspofungin (0.5 µg ml⁻¹) and micafungin (0.5 µg ml⁻¹). The MIC of fluconazole (16 µg ml⁻¹) was only in the dose-dependent range for one isolate (Table 2).

DISCUSSION
Late-onset fungal sepsis continues to be one of the leading causes of morbidity and mortality in neonates, in spite of considerable advances in neonatal care [15]. Although traditional *Candida* species are known to be the commonest aetiological agents, recent publications have emphasized the increasing importance of unusual pathogens [16, 17]. We report an outbreak of fungal sepsis in neonates admitted to a NICU that was caused by one such rare pathogen, *P. kudriavzevii* (a teleomorph of *Candida krusei*). To the best of our knowledge, this is first report of neonatal sepsis due to *P. kudriavzevii*.

### Table 2. *In vitro* antifungal susceptibility data (MIC, µg ml⁻¹) for *P. kudriavzevii* isolates against eight antifungal agents

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Range</th>
<th>GM</th>
<th>MIC₅₀</th>
<th>MIC₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>1–1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>2–16</td>
<td>13</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.25–0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.25–0.5</td>
<td>0.29</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>0.25–1</td>
<td>0.47</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>0.25–0.5</td>
<td>0.47</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Micafungin</td>
<td>0.25–0.5</td>
<td>0.47</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>0.25–0.5</td>
<td>0.47</td>
<td>0.5</td>
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GM, geometric mean; MIC, minimum inhibitory concentration.
Yeast species of the genus *Pichia* are widely distributed; in nature, e.g. in soil, fresh water, tree exudates, insects, plants and fruits, and also as contaminants in a variety of foods and beverages. Some *Pichia* species contribute desirable effects in the early stages of wine fermentation, in several types of brine and in different types of cheese, while other species are increasing in importance as opportunistic pathogens [18].

Unfamiliarity with the causative organism is known to impede its accurate identification. Some studies have emphasized that most of the uncommon yeasts are wrongly identified using conventional methods and this may have a bearing on treatment and clinical management [17, 19, 20].

In this study, the first case of late-onset fungal sepsis was observed in a 4-day-old preterm neonate with very low birth weight. In total, nine neonates were diagnosed with late-onset fungal sepsis due to *P. kudriavzevii* over a span of 2.5 weeks. All of the affected neonates had one or more of the common risk factors – the majority of the neonates were preterm, LBW/VLBW, had been put on a ventilator (two babies), had been admitted to the NICU before the onset of fungaemia (two babies), or had been put on broad-spectrum antibiotics before fungaemia developed (four babies) (Table 1). Most of the published data for fungal sepsis in neonates in the literature also report similar (four babies) (Table 1). Most of the published data for fungal sepsis in neonates in the literature also report similar

The time gap between the cases was on average 2–3 days, indicating rapid spread. The nine neonates were admitted to all three parts of the NICU. The first case was from the inborn NICU, while the other eight neonates were admitted to the outborn and HD NICUs, indicating common nosocomial modes of transmission. The HCWs in the NICU perform duties in all parts of the NICU on rotation, so a likely mode of transmission might be hands of HCWs. In spite of thorough and detailed environmental surveillance, we could not trace the source of infection. Other studies also reported that the source of the infection could not be identified [22, 23]. The samples from the hands of all the HCWs were negative, with the exception of a sample from one member of the nursing staff, which yielded yeast-like growth. This was identified as *W. anomalous*.

As antifungal drug susceptibility testing was not available at the hospital, all of the nine patients were advised treatment with voriconazole (4 mg/kg/day). Of these nine patients, seven were treated with voriconazole and responded to treatment. Two neonates were taken home against medical advice and could not be followed up. With no data or guidelines available for the treatment of *P. kudriavzevii* infection, this study shows promising results for the treatment of *P. kudriavzevii* fungaemia in neonates with voriconazole.

Limitation of the study was the failure to identify the source of the outbreak and also the mode of transmission. The outbreak was controlled after the implementation of stringent infection control measures, such as strict hand washing, the isolation of infected babies, meticulous care of medical devices and the use of appropriate personal protective equipment (PPE). Although designating dedicated (1:1) HCWs for the care of neonates would have been ideal, it was not practically feasible.

In conclusion, the study reports a rare emerging yeast, *P. kudriavzevii*, as a causative agent of fungal sepsis in neonates admitted to a NICU for the first time. Isolation of an unusual pathogen from inpatients with risk factors should raise the alarm for a possible outbreak. Performing antifungal susceptibility testing is important, as different susceptibility profiles will have clinical implications. As in other investigations [24, 25] this study emphasizes the importance of molecular methods which help in precise identification and determining the clonality of the aetiological agents involved in an outbreak.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Institutional ethical clearance was obtained.

References


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