Molecular screening for Candida orthopsilosis and Candida metapsilosis among Danish Candida parapsilosis group blood culture isolates: proposal of a new RFLP profile for differentiation

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Candida orthopsilosis and Candida metapsilosis are recently described species phenotypically indistinguishable from Candida parapsilosis. We evaluated phenotyping and molecular methods for the detection of these species among 79 unique blood culture isolates of the C. parapsilosis group obtained during the years 2004–2008. The isolates were screened by PCR amplification of the secondary alcohol dehydrogenase-encoding gene (SADH) followed by digestion with the restriction enzyme BanI, using C. parapsilosis ATCC 22019, C. orthopsilosis ATCC 96139 and C. metapsilosis ATCC 96144 as controls. Isolates with RFLP patterns distinct from C. parapsilosis were characterized by sequence analysis of the ITS1–ITS2, 26S rRNA (D1/D2) and SADH regions. Restriction patterns for the 3 species with each of 610 restriction enzymes were predicted in silico using 12 available sequences. By PCR-RFLP of the SADH gene alone, four isolates (5.1 %) had a pattern identical to the C. orthopsilosis reference strain. Sequence analysis of SADH and ITS (internal transcribed spacer) regions identified two of these isolates as C. metapsilosis. These results were confirmed by creating a phylogenetic tree based on concatenated sequences of SADH, ITS and 26S rRNA gene sequence regions. Optimal differentiation between C. parapsilosis, C. metapsilosis and C. orthopsilosis was predicted using digestion with NlaIII, producing discriminatory band sizes of: 131 and 505 bp; 74, 288 and 348 bp; and 131, 217 and 288 bp, respectively. This was confirmed using the reference strains and 79 clinical isolates. In conclusion, reliable discrimination was obtained by PCR-RFLP profile analysis of the SADH gene after digestion with NlaIII but not with BanI. C. metapsilosis and C. orthopsilosis are involved in a small but significant number of invasive infections in Denmark.

Abbreviations: AFLP, amplification fragment length polymorphism; AFST–EUCAST, Antifungal Susceptibility Testing Subcommittee–European Committee on Antimicrobial Susceptibility Testing; ATCC, American Type Culture Collection; ITS, internal transcribed spacer; RAPD, randomly amplified polymorphic DNA. The GenBank/EMBL/DDBJ accession numbers for the SADH sequences reported in this study are FJ746046–FJ746069.
INTRODUCTION

Candida parapsilosis is one of the five most common yeasts involved in invasive candidiasis and is particularly common in neonates, in catheter-associated candidaemia and in association with intravenous hyperalimentation (Krcmery & Barnes, 2002). In Denmark, it is involved in 4% of the fungaemia cases (Arendrup et al., 2008). C. parapsilosis is normally susceptible to most antifungal compounds but elevated MICs for the echinocandin agents are consistently reported for this species due to an intrinsic mutation in the FKS hot spot (Garcia-Effron et al., 2008). Susceptibility differences within the C. parapsilosis group could affect therapeutic choices (van Asbeck et al., 2008).

C. parapsilosis is a complex of three genetic groups (I, II and III) according to the results from genotypic methods, including randomly amplified polymorphic DNA (RAPD) analysis (Lehmann et al., 1992), karyotyping (Lott et al., 1993), multicilous enzyme electrophoresis, ribosomal internal transcribed spacer (ITS) sequencing (Lin et al., 1995), DNA reassociation analysis (Roy & Meyer, 1998), DNA topoisomerase II gene sequencing (Kato 1995), DNA reassociation analysis (Roy & Meyer, 1998), internal transcribed spacer (ITS) sequencing (Lin et al., 1993), multilocus enzyme electrophoresis, ribosomal species

METHODS

Isolates. A total of 79 clinical C. parapsilosis group isolates and 3 reference strains, C. parapsilosis ATCC 22019, C. orthopsilosis ATCC 96139 and C. metapsilosis ATCC 96144, were used in this study. Clinical strains were obtained from Danish patients with candidaemia during 2004–2008 (Arendrup et al., 2008). Species identification was based on colony colour and morphology on CHROMagar, microscopic morphology on cornmeal agar and use of a commercial system (ATB ID32C). The isolates had been stored in glycerol/water at −80 °C. All strains were subcultured on CHROMagar and incubated at 37 °C for 48 h for reconfirmation of the identification as C. parapsilosis sensu lato. Susceptibility testing was performed according to the European Committee on Antimicrobial Susceptibility Testing Edelf 7.1 guidelines (AFST-EUCAST, 2008).

PCR. Reference and clinical strains were subjected to amplification of the SADH gene. A small amount (about 0.5–1 μl) of a single fresh colony was added directly, and without prior DNA extraction (Mirmehdi et al., 2007) to the PCR premix, which contained 0.5 μM of the forward (5'-GGT GAT GCT GGA TTG T-3') and reverse (5'-CAA TGC CAA ATC TCC CAA-3') primers (Tavanti et al., 2005), 400 μM deoxynucleoside triphosphate mix, 2.5 μM magnesium-free buffer, 1.5 mM MgCl2 and 1.25 U Taq DNA polymerase (Sigma-Aldrich) in a final volume of 25 μl. A negative control (water) was included in all PCR experiments. Conditions for PCR amplification were: 94 °C for 7 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 60 s and elongation at 72 °C for 90 s; and a final extension step at 72 °C for 5 min.

RFLP. The SADH PCR products were digested for 120 min with the restriction enzyme BanI or NlaIII (Fermentas), in a 15 μl reaction volume containing 0.5 μl (5 U) enzyme, 1.5 μl 10× buffer (supplied with the enzyme), 5 μl PCR product and 8 μl molecular grade water.

Electrophoresis. A total of 5 μl amplified DNA and 8 μl digested DNA product were separated by electrophoresis on 1.5% and 2% agarose gels, respectively. The electrophoresis buffer was TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3). A 100 bp DNA ladder was used as a molecular size marker. DNA bands were visualized by UV transillumination and photographed.

Sequencing. The following forward and reverse primer pairs were used for amplification and sequencing of the target DNA regions: 5'-TCC GTA GGT GAA CCT GCG G-3' and 5'-TCC TCC GAT TAT TGA TAT GC-3' for the ITS1–ITS2 domain of the large subunit of rDNA gene (26S rDNA) (Fell, 1993); 5'-GCA TAT CAA TAA GCG GAG GAA AAG-3' and 5'-GGT CCG TGT TTC ACG GAG-3' for the D1/D2 domain of the large subunit of rDNA (26S rDNA) (Fell, 1993); and 5'-GGT GAT GCT GGA TTG T-3' and 5'-CAA TGC CAA ATC TCC CAA-3' for the SADH gene (Tavanti et al., 2005). Sequencing was performed in all cases by the company MWG using a BigDye terminator cycle sequencing kit and an ABI-3730 genetic analyzer.

Sequence analysis. Forward and reverse sequences were aligned and compared to ensure agreement, and then subjected to nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and DNASIS multiple alignment. Four separate phylogenetic analyses of sequence data were conducted: three using each individual SADH, ITS1–ITS2 and 26S rRNA gene sequence data for sequences determined in the present study and employing the same Candida dubliniensis data for the outgroup; and one using concatenated SADH + ITS1–ITS2 + 26S rRNA data representing the total nucleotide variation in all three regions of the tested strains, with C. dubliniensis as the outgroup. The analyses were run using the Bayesian inference method with the program MrBayes v3.1.2. The evolutionary distance was calculated by employing the general time reversible evolutionary model (nst=6), which utilizes a gamma-shaped variation in mutation rates between codons (rate=gamma). Posterior probabilities were calculated via 2000000 generations (ngen=2000000, burnin=20000) using the Monte Carlo
Markov chain method and four simultaneous tree building chains (nchains=4), with every 100th tree saved (samplefreq=100).

**Designing a new RFLP profile.** The SADH sequences obtained in this study (GenBank accession nos FJ746046–FJ746069) and all available SADH sequences deposited in GenBank for isolates belonging to the *C. parapsilosis* group (http://www.ncbi.nlm.nih.gov/) were compared and analysed by DNASIS software. The cutting sites of enzymes listed in the DNASIS software were predicted and evaluated for potential to discriminate between *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*.

**RESULTS AND DISCUSSION**

The colony colour of the 79 *C. parapsilosis sensu lato* isolates was white-cream to pale pink on CHROMagar Candida with slight, but not species-specific, differences among the isolates. ITSs and the 26S rRNA gene sequence are well-known targets for most of the taxonomic studies in nearly all microorganisms. They are the basis of routine and research identification of fungal pathogens and for recognizing new species (Iwen *et al.*, 2002). The SADH gene has been the basis of sequencing analysis or BanI-RFLP for identification of the *C. parapsilosis* group since the new species were described in 2005 by Tavanti and colleagues (Tavanti *et al.*, 2005). Thus these genes were chosen for comparative studies of the isolates. The SADH gene was successfully amplified in all the clinical isolates, as well as the reference strains, and a single sharp band with a size of approximately 720 bp was seen for all samples. Digestion of PCR products by the restriction enzyme BanI resulted in three different band patterns (Fig. 1). A double band pattern (approx. 520 and 200 bp in size) was observed for 75/79 clinical isolates. A single band representing the absence of a cutting site for BanI was seen for *C. orthopsilosis* ATCC 96139 (Fig. 1, lane 2) and four clinical isolates (Fig. 1, lanes 4, 5, 10 and 21). These results were reproduced three times for these strains in order to exclude any errors in the enzymic reaction. Finally, as expected, a three band pattern (approx. 390, 190 and 100 bp) was observed for *C. metapsilosis* ATCC 96144 (Fig. 1, lane 1) (a fourth 40 bp band is not visible by gel electrophoresis). Thus, according to the digestion patterns described previously (Tavanti *et al.*, 2005), 75 blood culture isolates were identified as *C. parapsilosis*, 4 as *C. orthopsilosis* (strains W36279, T27682, T19754 and F10552), and none as *C. metapsilosis* (Table 1).

For confirmation of the species identification obtained by SADH-RFLP, each of the three reference strains, the four blood culture isolates identified as *C. orthopsilosis* by RFLP and three randomly selected *C. parapsilosis* isolates, were sequenced for the three DNA markers: SADH, the entire ITS1–ITS2 region and 26S rRNA (D1/D2) gene sequence. Forward and reverse sequences determined were completely compatible. The sequences were subjected to a nucleotide BLAST search and sequence analysis of the SADH and ITS1–ITS2 regions, which resulted in identification of two isolates (strains T27682 and F10552) as *C. orthopsilosis* (Fig. 1, lanes 5 and 21) and two isolates (strains T19754 and W36279) as *C. metapsilosis* (Fig. 1, lanes 4 and 10) (Table 1). There was no cutting site for the enzyme BanI at in silico digestion of the sequences of these isolates. For the American Type Culture Collection (ATCC) reference strains and three randomly selected *C. parapsilosis* isolates sequence analysis of the SADH gene and ITS region confirmed the species identification obtained by SADH-RFLP (Table 1). Sequence analysis of the 26S rRNA (D1/D2) region failed to differentiate *C. parapsilosis* from *C. metapsilosis* and *C. orthopsilosis*, even for the *C. orthopsilosis* and *C. metapsilosis* ATCC reference strains (Table 1).

To evaluate the nucleotide variation, the sequences obtained in this study were also subjected to multiple alignment and analysis of phylogenetic trees, separately for each region and also concatenated for all regions. A total of 592 nt for the SADH gene, 434 nt for the ITS1–ITS2 region, 591 nt for 26S rRNA (data not shown) and 1617 nt for the concatenated analysis were used. As shown in Fig. 2, two of four clinical isolates that initially were identified as *C. orthopsilosis* by SADH PCR-RFLP, grouped with *C. metapsilosis* ATCC 96144. The SADH sequence was identical when compared to the two Danish *C. metapsilosis* isolates and did not contain a BanI cutting site. The ATCC 96144 *C. metapsilosis* SADH sequences were 95% similar to the two Danish isolates, and the reference strain contained a BanI cutting site.

With the objective of designing a better SADH-RFLP profile for species separation and identification of the *C. parapsilosis* group, all available sequences of the SADH gene, including the sequences obtained from the present study and sequences previously deposited in GenBank, were analysed and restriction patterns were predicted for

![Fig. 1. Agarose gel electrophoresis of SADH PCR products digested by the restriction enzyme BanI. Lane 1, C. metapsilosis ATCC 96144; lane 2, C. orthopsilosis ATCC 96139; lane 3, C. parapsilosis ATCC 22019; lanes 4–30, blood culture isolates; lane M, 100 bp molecular size marker. Note the single band pattern in lanes 4, 5, 10 and 21, as well as for the reference strain C. orthopsilosis (lane 2). Note also the slightly smaller band size for samples 4 and 10 (which were eventually identified as C. metapsilosis).](image-url)
Table 1. Molecular identification of 79 clinical C. parapsilosis sensu lato blood culture isolates and 3 reference strains using phenotyping and a complement of molecular methods

<table>
<thead>
<tr>
<th>Strain</th>
<th>SADH PCR-RFLP with BanI</th>
<th>SADH gene sequencing</th>
<th>ITS1–ITS2 sequencing</th>
<th>26S rRNA (D1/D2) gene sequencing</th>
<th>SADH PCR-RFLP with NlaIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 22019</td>
<td>C. parapsilosis</td>
<td>C. parapsilosis</td>
<td>C. parapsilosis</td>
<td>C. parapsilosis</td>
<td>C. parapsilosis</td>
</tr>
<tr>
<td>ATCC 96144</td>
<td>C. metapsilosis</td>
<td>C. metapsilosis</td>
<td>C. metapsilosis</td>
<td>C. orthopsilosis/metapsilosis</td>
<td>C. metapsilosis</td>
</tr>
<tr>
<td>ATCC 96139</td>
<td>C. orthopsilosis</td>
<td>C. orthopsilosis</td>
<td>C. orthopsilosis</td>
<td>C. orthopsilosis/metapsilosis</td>
<td>C. orthopsilosis</td>
</tr>
<tr>
<td>W36279 &amp; T19754</td>
<td>C. orthopsilosis</td>
<td>C. metapsilosis</td>
<td>C. metapsilosis</td>
<td>C. orthopsilosis/metapsilosis</td>
<td>C. orthopsilosis</td>
</tr>
<tr>
<td>T27682 &amp; F10552</td>
<td>C. orthopsilosis</td>
<td>C. orthopsilosis</td>
<td>C. orthopsilosis</td>
<td>C. orthopsilosis/metapsilosis</td>
<td>C. orthopsilosis</td>
</tr>
<tr>
<td>Remaining 75 blood isolates</td>
<td>C. parapsilosis</td>
<td>Not done*</td>
<td>Not done*</td>
<td>Not done*</td>
<td>C. parapsilosis</td>
</tr>
</tbody>
</table>

*Three of these blood isolates underwent sequencing of the SADH gene and of the 26S rRNA (D1/D2) gene region, and one blood isolate underwent ITS1–ITR2 sequencing, confirming the isolates to be C. parapsilosis.

610 (almost all available) restriction enzymes. The most applicable enzymes were MwoI, Hpy8I, Mfel, BstENII, AfaI, AlwI and NlaIII, but only NlaIII (FatI) and BstENII were predicted to have cutting sites for all three species. Comparing the predicted fragment sizes of NlaIII (FatI) and BstENII, NlaIII was superior, due to having the greatest difference in the main fragment sizes between the species. For NlaIII the following fragment sizes were found: C. parapsilosis – 131 and 505 bp, C. metapsilosis – 74, 288 and 348 bp, and C. orthopsilosis – 131, 217 and 288 bp. In comparison the following fragment sizes were found for BstENII: C. parapsilosis – 141 and 576 bp, C. metapsilosis – 158 and 363 bp. The SADH PCR products of all blood culture isolates and reference strains were digested with NlaIII (examples are shown in Fig. 3). The fragment patterns were as expected and in agreement with the SADH gene sequence data. No additional C. orthopsilosis or C. metapsilosis isolates were found among clinical isolates by this new technique. Finally, susceptibility to amphotericin B, caspofungin, fluconazole, itraconazole and voriconazole were compared. Given as MICs for the two C. metapsilosis isolates, MICs for the two C. orthopsilosis isolates and MIC50 (range) for the 75 C. parapsilosis isolates, the values (μg ml⁻¹) were as follows: amphotericin B, 0.5 and 0.25, 0.5 and 0.5, and 0.5 (0.125–1), respectively; caspofungin,
0.5 and 1, 0.5 and 0.5, and 2 (0.5–4), respectively; fluconazole, 2 and 0.25, 0.5 and 1, and 1 (0.5–8), respectively; itraconazole, 0.125 and ≤0.032, ≤0.032 and 0.125, 0.064 (≤0.032–0.125), respectively; and voriconazole, 0.064 and ≤0.032, ≤0.032 and 0.064, and ≤0.032 (≤0.032–0.125), respectively. Thus, no significant differences were observed.

In this study we have reported the prevalence of the recently described species \( C. \) orthopsilosis and \( C. \) metapsilosis in a well-defined sample of blood culture isolates from Denmark and a new low cost PCR-RFLP method for the reliable separation of the \( C. \) parapsilosis group into the three species. Overall, 2.5% of the isolates phenotypically identified as \( C. \) parapsilosis were \( C. \) orthopsilosis and 2.5% were \( C. \) metapsilosis. Table 2 summarizes the frequencies of \( C. \) orthopsilosis and \( C. \) metapsilosis in other studies of \( C. \) parapsilosis sensu lato. In the majority of studies, including all those with more than 100 isolates, 88% or more of the isolates were confirmed to be \( C. \) parapsilosis in agreement with the current findings. In Portugal, Spain and Denmark similar proportions of \( C. \) orthopsilosis and \( C. \) metapsilosis isolates were detected.

### Table 2. Summary of published studies on identification methods with distribution of \( C. \) parapsilosis, \( C. \) orthopsilosis and \( C. \) metapsilosis

<table>
<thead>
<tr>
<th>Method used for differentiation</th>
<th>Source of specimens</th>
<th>No. of isolates (%) by species</th>
<th>Country or region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( C. ) parapsilosis</td>
<td>( C. ) orthopsilosis</td>
<td>( C. ) metapsilosis</td>
</tr>
<tr>
<td>MLST, RAPD-PCR, SADH RFLP</td>
<td>Various clinical sources</td>
<td>20 (74.1 %)</td>
<td>7 (25.9 %)</td>
<td>0</td>
</tr>
<tr>
<td>Direct PCR-sequencing of BacTec</td>
<td>Blood and other clinical sources</td>
<td>8 (88.9 %)</td>
<td>1 (11.1 %)</td>
<td>0</td>
</tr>
<tr>
<td>ITS sequencing, RAPD PCR, phenotyping data</td>
<td>Blood and other clinical sources</td>
<td>18 (90 %)</td>
<td>0</td>
<td>2 (10 %)</td>
</tr>
<tr>
<td>SADH-RFLP, AFLP</td>
<td>Various clinical sources</td>
<td>277 (95.5 %)</td>
<td>13 (4.5 %)</td>
<td>0</td>
</tr>
<tr>
<td>SADH-RFLP, AFLP</td>
<td>Various clinical sources</td>
<td>375 (94.9 %)</td>
<td>5 (6.4 %)</td>
<td>6 (7.7 %)</td>
</tr>
<tr>
<td>ITS sequencing</td>
<td>Blood</td>
<td>67 (85.9 %)</td>
<td>6 (7.7 %)</td>
<td>3 (4.4 %)</td>
</tr>
<tr>
<td>SADH-RFLP</td>
<td>Invasive clinical samples</td>
<td>1762 (92.1 %)</td>
<td>117 (6.1 %)</td>
<td>34 (1.8 %)</td>
</tr>
<tr>
<td>ITS-sequencing, RAPD PCR</td>
<td>Blood</td>
<td>29 (70.7 %)</td>
<td>10 (24.4 %)</td>
<td>2 (4.9 %)</td>
</tr>
<tr>
<td>PCR with specific primers, SADH-RFLP, D1/D2 and ITS sequencing</td>
<td>Blood (66) and other clinical sources (48)</td>
<td>109 (95.6 %)</td>
<td>5 (4.4 %)</td>
<td>0</td>
</tr>
<tr>
<td>SADH-RFLP</td>
<td>Clinical and environmental sources</td>
<td>160 (94.67 %)</td>
<td>4 (2.37 %)</td>
<td>5 (2.96 %)</td>
</tr>
<tr>
<td>ITS sequencing</td>
<td>Blood</td>
<td>126 (88.1 %)</td>
<td>13 (9.1 %)</td>
<td>4 (2.8 %)</td>
</tr>
<tr>
<td>SADH-RFLP, SADH, ITS and 26S sequencing</td>
<td>Blood</td>
<td>75 (95 %)</td>
<td>2 (2.5 %)</td>
<td>2 (2.5 %)</td>
</tr>
</tbody>
</table>

NI, Not indicated.
(Gomez-Lopez et al., 2008; Silva et al., 2009), while C. orthopsilosis was more frequent than C. metapsilosis in a worldwide study with almost 2000 isolates (Lockhart et al., 2008) and in two smaller studies from Malaysia and Kuwait (Tay et al., 2009; Asadzadeh et al., 2009). To what extent these observations reflect true differences compared to method-dependent effects remains to be elucidated. Some studies have used SADH-RFLP with BanI, which we found unreliable for identifying C. metapsilosis. Some studies have used PCR sequencing of the D1/D2 region of 26S rRNA gene; however, by sequence analysis (Table 1) including the use of phylogenetic trees, we showed that this region is not a suitable target for separation of the species within the C. parapsilosis group. The 26S rRNA gene sequence is generally a highly conserved region, particularly in yeasts (Iwen et al., 2002). Thus it is foreseeable that 26S rRNA (D1/D2) gene sequence may not be an optimal marker for differentiating closely related species.

Since the description of C. orthopsilosis and C. metapsilosis in 2005 (Tavanti et al., 2005), several methods have been proposed to identify and differentiate these species from C. parapsilosis sensu stricto. Tavanti et al. (2005) proposed PCR amplification of the SADH gene followed by restriction digestion with BanI, and used amplification fragment length polymorphism (AFLP) for identification and genotyping of about 400 isolates previously classified as C. parapsilosis. AFLP was shown to identify C. orthopsilosis (Tavanti et al., 2007) and C. metapsilosis (Hensgens et al., 2009) to the species level, and to be an efficient genotyping tool as well, delineating intraspecific genetic relatedness. Campa et al. (2008) developed an oligonucleotide microarray based on the arrayed-primer extension technique to simultaneously identify pathogenic fungi. They designed probes complementary to the ITS1 and ITS2 region for identification of 24 species, including C. parapsilosis, C. orthopsilosis and C. metapsilosis. Based on the major DNA fragments of the RAPD analysis profiles, Tay et al. (2009) identified the isolates as types P1 to P5. Sequence analysis of the ITS region of the isolates identified RAPD type P1 as C. parapsilosis, P2 and P3 as C. orthopsilosis, and P4 as C. metapsilosis. van Asbeck et al. (2008) reported the correlation of AFLP genotyping with ITS sequence, RAPD and multilocus sequencing for the C. parapsilosis group. According to Borman et al. (2009) pyrosequencing analysis of 20 nucleotides of the ITS2 region rapidly and robustly distinguished the three species. Finally, Asadzadeh et al. (2009) introduced primers derived from unique sequences within the ITS1–5.8S rDNA–ITS2 region for the purpose of differentiating the three species. All these methods have the advantages of a sequencing-based approach, but are time consuming and labour intensive, and some are expensive.

In our hands the SADH PCR-RFLP with the enzyme BanI successfully separated C. parapsilosis sensu stricto from the two new species, but the band patterns of C. orthopsilosis and C. metapsilosis were so similar that misclassification of C. metapsilosis as C. orthopsilosis must be expected in a routine setting. As PCR-RFLP is an attractive method due to low cost, we carefully analysed the SADH region in the C. parapsilosis complex for differences that predicted individual RFLP patterns and confirmed that the use of SADH-PCR followed by digestion by NlaIII could be a more reliable method for differentiating the three species. Further studies using this method and a higher number of C. metapsilosis and C. orthopsilosis isolates are needed to confirm the robustness of the method.

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