Objectives: Bovine mammary protothecosis is a serious pathology that entails high economic losses in the dairy industry. The disease, the frequency of which has recently been increasing worldwide, is caused by unicellular, achlorophyllous, yeast-like algae of two species: *Prototheca zopfii* and *Prototheca blaschkeae*. The objective of this study was to investigate the in vitro activity of a panel of conventional antifungal drugs against *Prototheca* spp. isolates.

Methods: A total of 144 *P. zopfii* genotype 2 and *P. blaschkeae* strains isolated from milk of mastitic cows were subjected to drug susceptibility testing by Etest methodology.

Results: Five out of ten antifungal drugs tested exhibited no activity against *Prototheca* spp. isolates. The best activity against *Prototheca* spp. was demonstrated by amphotericin B (MIC90 of 1.5 mg/L). The MICs differed significantly (*P*, 0.01) between *P. zopfii* genotype 2 and *P. blaschkeae*, with the latter species being more susceptible to amphotericin B and azoles. Marked differences (*P*, 0.05) in azole and amphotericin B activities were noted among *Prototheca* spp. isolates originating from different European countries. Based on the correlation coefficients, a considerable cross-interaction was found among MICs of azoles and between MICs of azoles and amphotericin B for *Prototheca* spp. (*P*, 0.03).

Conclusions: This study represents the largest, cross-European evaluation of antifungal activity against *Prototheca* spp. to date. The activity of amphotericin B against *Prototheca* spp. validates its potential use as a therapeutic agent against bovine protothecosis. For laboratory testing of drug activity against *Prototheca* spp., the Etest method is encouraged, due to its technical simplicity, rapidity and high intra- and inter-laboratory reproducibility.

Keywords: yeast-like algae, amphotericin B, azoles, drug resistance, genotypes

Introduction

Yeast-like, colourless, unicellular algae of the genus *Prototheca* are the only known plant organisms to possess pathogenic potential for both humans and animals. At present, four different species are recognized, namely, *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca stagnora* and *Prototheca ulmea*.1 Recently, *P. zopfii* has been divided, on the basis of 18S rRNA sequence analysis, into three distinct genotypes, of which *P. zopfii* genotype 3 has attained the status of a new species, *Prototheca blaschkeae*.2

Whereas human protothecosis is caused by *P. wickerhamii* and *P. zopfii*, the causative agents of bovine mastitis (the most frequently observed form of animal protothecosis) are *P. zopfii* and *P. blaschkeae*.1,3 Bovine mammary protothecosis has been recognized globally, but only recently has gained significant attention.

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attention from the veterinary and agricultural sectors, due to its increasingly widespread occurrence. The disease most often presents as a chronic condition leading to a progressive decrease in milk yield and deterioration in milk quality. This results in the depressed productivity of dairy cows and consequently incurs important economic losses to the dairy industry.1,3,4

Studies have frequently demonstrated that a number of antimicrobial drugs exhibit low, or even lack of, activity against Prototheca species.5–8 However, most of the evaluations performed so far have suffered from small sample sizes and lack of statistical power.6–8 Consequently, a very fragmentary picture about the activity of antimicrobial drugs against Prototheca spp. is currently available.

The purpose of this work was to investigate the in vitro activity of a panel of conventional antifungal agents against an ample European collection of P. zapfii and P. blaschkeae isolates, using the Etest methodology.

Materials and methods

A total of 144 Prototheca spp. isolates (113 P. zapfii genotype 2 and 31 P. blaschkeae) were used in the study. The isolates were originally retrieved from milk samples of individual cows affected by clinical and subclinical mastitis maintained in different dairy herds in Germany, Italy, Poland, Portugal, Belgium and France. Two reference strains were obtained from the Culture Collection of Algae (SAG), University of Göttingen, Germany: P. zapfii genotype 2 SAG 2021 and P. blaschkeae SAG 2064. Moreover, three quality control strains were used to validate the performance of the Etest assay: Candida krusei DBVPG 7235 (ATCC 6258) and Candida parapsilosis DBVPG 6150 (ATCC 22019) from the Industrial Yeasts Collection DBVPG, Italy, and Candida albicans ATCC 90028 from the ATCC (USA).

Ten antifungal agents were evaluated: amphotericin B, fluconazole, itraconazole, ketoconazole, posaconazole, voriconazole, anidulafungin, caspofungin and micafungin. The Etest strips were purchased from bioMérieux (France); they contained increasing concentration gradients of the antifungals, from 0.002 to 32 mg/L for all drugs tested, except for fluconazole (from 0.016 to 256 mg/mL). The strips were stored at −20°C before use.

Aliquots of 24–48 h Prototheca cells were suspended in sterile 0.85% NaCl aqueous solutions to a turbidity equivalent to that of a 0.5 McFarland standard (approx. 10⁶ cfu/mL). The culture medium used was RPMI 1640 (Sigma, USA) without sodium bicarbonate, supplemented with 2% (w/v) glucose, adjusted to pH 7.0 with 0.165 M MOPS and solidified with 2% (w/v) agar. The RPMI agar plates were inoculated by dipping a sterile cotton swab into the inoculum and evenly streaking the entire surface of each plate in three directions. After allowing the plates to dry completely, Etest strips were applied to the surfaces. Plates were incubated at 35°C, with readings taken after 24 and 48 h. Each MIC was measured as the point where the inhibition ellipse intersected the Etest strip gradient scale. All tests were performed in duplicate.

No universally accepted guidelines specific for Prototheca species applicable in the interpretation of drug susceptibility testing are at present available. Non-parametric methods of statistical analysis were applied. Differences in the MIC values of different antifungal agents between P. zapfii genotype 2 and P. blaschkeae populations and among the Prototheca spp. isolated from distinct countries were compared by the Mann–Whitney U-test and the Kruskal–Wallis test, respectively. The existence of cross-interactions among MIC profiles of different antifungals was evaluated using the Spearman correlation coefficient test. Statistical significance was set at P < 0.05. All calculations were performed using the STATISTICA software package (ver. 8.0; StatSoft Inc., Tulsa, USA).

Results and discussion

The MIC ranges, MIC geometric means (GMs), MIC₅₀ and MIC₉₀ of 10 antifungal drugs for the Prototheca isolates are reported in Table 1. All the Prototheca isolates grew in the presence of 32 mg/L fluconazole, anidulafungin, caspofungin and micafungin, and in the presence of 256 mg/L fluconazole (Table 1). The activities of ketoconazole, itraconazole, posaconazole and voriconazole were strain-dependent, with 61.8%, 56.9%, 48.6% and 31.9% of Prototheca isolates growing at the highest concentration (32 mg/L) of the four azoles, respectively. The best activity against Prototheca spp. was demonstrated by amphotericin B; growth of more than 97% of the isolates was inhibited at a drug concentration of <2 mg/L (MIC₅₀ and MIC₉₀ of 0.5 and 1.5 mg/L, respectively).

Overall, when compared with azoles, fluconazole and echinocandins, amphotericin B exhibited the highest activity against Prototheca isolates, thus corroborating and expanding the

Table 1. MIC ranges, MIC GMs, MIC₅₀ and MIC₉₀ of antifungal agents used in this study for P. zapfii gen. 2 and P. blaschkeae isolates

<table>
<thead>
<tr>
<th>MIC (mg/L)</th>
<th>P. zapfii genotype 2 (n = 113)</th>
<th>P. blaschkeae (n = 31)</th>
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<tr>
<td></td>
<td>range</td>
<td>GM</td>
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<tr>
<td>Fluconazole</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<tr>
<td>Fluconazole</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>1.5–&gt;32</td>
<td>16.86</td>
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<tr>
<td>Ketoconazole</td>
<td>0.75–&gt;32</td>
<td>22.98</td>
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<tr>
<td>Posaconazole</td>
<td>0.5–&gt;32</td>
<td>9.33</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.75–&gt;32</td>
<td>8.21</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.19–3</td>
<td>0.63</td>
</tr>
<tr>
<td>Anidulafungin</td>
<td>&gt;32</td>
<td>&gt;32</td>
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<tr>
<td>Caspofungin</td>
<td>&gt;32</td>
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<td>Micafungin</td>
<td>&gt;32</td>
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findings from previous investigations. More importantly, the MIC profiles of P. zopfii genotype 2 and P. blaschkeae notably differed (P<0.01); the latter species was more susceptible to amphotericin B and to azoles, and thus could be more easily eradicated by using these drugs (Table 1). Interestingly, genotype-specific differences in drug activity against Prototheca isolates have been demonstrated previously: amphotericin B and itraconazole were more efficacious against P. zopfii genotype 1 isolates than against P. zopfii genotype 2 isolates.

The correlation coefficients (r) among MICs of five different drugs for Prototheca isolates were calculated. A high correlation was observed between amphotericin B–itraconazole, amphotericin B–ketoconazole and amphotericin B–voriconazole, as well as between every two azoles tested (r values between 0.19 and 0.80, P<0.03). This finding implicating the existence of cross-interactions among azoles and between azoles and amphotericin B against Prototheca spp. is in agreement with what has been observed in yeasts. Although the molecular mechanisms behind such cross-interactions are largely unknown, it has been speculated that increased expression of specific efflux pumps may play a definite role.

Finally, marked differences (P<0.05) inazole and amphotericin B activities against Prototheca strains were noted among isolates originating from different European countries (data not shown). This may be the consequence of epidemiological, environmental and technological factors (e.g. circulation of several distinct P. zopfii genotype 2 and P. blaschkeae clones in the different countries, different climatic and ecological conditions, and heterogeneous prophylaxis and therapy protocols applied in dairy herds for milk sampling etc.), which could have affected the phenotype of the clusters of isolates.

Mechanisms responsible for the differential activities exhibited by the drugs against Prototheca isolates remain obscure and can only be conjectured from the experimental evidence gathered for yeasts. The lack of activity of flucytosine against Prototheca spp. is rather of an intrinsic character, since flucytosine has seldom been used in the treatment of mammary protothecosis; similarly to Candida spp., this may result from impaired uptake of the drug into the cell. Likewise, echinocandins were ineffective against Prototheca spp. One explanation might be the lack of β-1,3-D-glucans (the synthesis of which is inhibited by echinocandins) in the cell wall of the algae. In contrast, the activity of polyenes, such as amphotericin B, and of azoles relates to the presence of ergosterol (the principle target of their activities) in the cell membranes of Prototheca species. The different modes of action of the polyenes and azoles may explain their different efficacies against the Prototheca spp. Whereas polyenes bind to ergosterol and cause permeability of the membranes by pore formation, resulting in osmotic imbalance and cell death, azoles inhibit the enzyme 14α-lanosterol demethylase, leading to the blockage of ergosterol synthesis.

In conclusion, this study is believed to represent the first European, multicentre, large-scale investigation of the in vitro activity of antifungal agents of different classes against Prototheca spp. isolates. The importantly high activity of amphotericin B against Prototheca spp. validates its potential use as a therapeutic agent for bovine protothecosis. Nevertheless, further studies are needed to assess more accurately the in vitro potency of amphotericin B and to determine its clinical efficiency. For laboratory testing of drug activity against Prototheca spp., the Etest method is strongly encouraged, due to its technical simplicity, robustness, rapidity and high reproducibility in intra- and inter-laboratory comparisons.

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This study was carried out as part of our routine work.

Transparency declarations
None to declare.

References