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Short Communication

Large-scale screening of the *in vitro* susceptibility of *Prototheca zopfii* towards polyene antibiotics

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A large-scale screening of the *in vitro* susceptibility of 105 strains of *Prototheca zopfii* to a panel of polyene antibiotics (amphotericin B, nystatin, pimaricin and filipin) was conducted. Strains studied were isolated from dairy-associated environments in five different localities. Groups 1–4 included strains recovered from four separate regions of Italy, while group 5 included isolates from Belgium. Amphotericin B and pimaricin exhibited the highest activity, with the MIC_{90} ranging from 4 and 8 μg/ml, respectively. On the other hand, the MIC_{90} of nystatin and filipin were from two to four times higher. Two strains were resistant to all four polyenes tested. The above results are compared with those in the literature and the importance of carrying out large-scale screening surveys to assess polyene susceptibility patterns within the species *P. zopfii* is discussed.

Keywords *Prototheca zopfii*, antimicrobial susceptibility, polyenes, bovine mastitis

Introduction

Within the genus *Prototheca*, *Prototheca wickerhamii* and *Prototheca zopfii* have been associated with both human [1] and animal [2,3] diseases. Mastitis caused by *P. zopfii* represent the primary clinical presentation of protothecosis in dairy cows [4,5]. In such cases, mammary gland infections caused by the fungus are rarely observed with clinical signs. All stages of lactation appear to be equally susceptible to infection (including dry cows), with the disease restricted to the udder or disseminating to the lymph nodes [4–6]. Outbreaks of bovine mastitis due to *P. zopfii* have been extensively described as a global problem [5,7–14].

It is well known that the antibiotic treatment of protothecosis is a key problem in veterinary medicine, because numerous studies have reported that *P. zopfii* is resistant to most antibiotics [5,6,15–19]. Culling of infected cows is usually recommended as the most effective prophylaxis practice. Accordingly, control measures generally involve the identification, removal and slaughter of infected animals, particularly when the disease is sporadically distributed within the herd. Additional measures for the control of *P. zopfii* infections (as well as that of other opportunistic udder pathogens) include the improvement of the hygienic conditions of herds and of milking equipment [4–6].

Polyene antibiotics currently used in human protothecosis therapy (amphotericin B and nystatin) have been shown to exhibit *in vitro* activity against *P. zopfii* [15,20]. However, a limited number of target strains (6 and 8, respectively) was studied. Besides, no additional polyene drugs (other than amphotericin B and nystatin) have been so far tested against this fungus. As a consequence, the literature provides only some limited information regarding the *in vitro* susceptibility

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of *P. zopfii* to members of this class of drugs. The present study reports the results of a large-scale survey of the *in vitro* susceptibility of *P. zopfii* strains to four polyene antibiotics (amphotericin B, nystatin, pimaricin and filipin).

Materials and methods

One hundred and five strains of *P. zopfii* were employed in this investigation. All were deposited in the Industrial Yeast Collection DBVPG of the University of Perugia (www.agr.unipg.it/dbvpg). Strains were isolated from bedding ($n=9$), drinking water ($n=2$), milking teats ($n=6$) and from the milk of infected cows ($n=88$). These isolates were obtained from five geographical locations: group 1 ($n=22$) from central Italy (Umbria region); group 2 ($n=23$) recovered in northwestern Italy (Piedmont region); group 3 ($n=23$) isolated in northern-central Italy (Emilia-Romagna region); group 4 ($n=23$) obtained from Northern Italy (Lombardy region); and group 5 ($n=14$) from Belgium (Mons). To the authors' knowledge, there was no contact between the animals in these different region/countries. One additional strain (labeled as NRRL Y-6868), obtained from the Culture Collection of the Agricultural Research Service, National Centre for Agricultural Utilization Research, Peoria, Illinois, USA, was used as a reference.

Four polyene antibiotics (amphotericin B, nystatin, pimaricin and filipin) purchased from Sigma-Aldrich (USA) were used in this study. Working solutions were prepared immediately before use in the susceptibility tests. In the absence of universally accepted procedures or interpretative criteria specific for *Prototheca* species, CLSI guidelines were followed [21]. Accordingly, minimum inhibitory concentrations (MICs) were determined in RPMI 1640 (Sigma-Aldrich) by using 96-well microtiter plates (Corning Inc., USA). In order to check the accuracy of the method, as proposed by CLSI guidelines [21], two quality control (QC) yeast strains were also used, i.e., *Candida parapsilosis* ATCC 22019 (DBVPG 6150) and *Candida krusei* ATCC 6258 (DBVPG 7235). All four polyene antibiotics were tested in duplicate at concentrations from 0.5 to 32 μg/ml. No discrepant results were obtained in more than 98% of the trials, but when they occurred, the test was repeated in triplicate. Only data exhibiting $\geq 66\%$ agreement for each isolate were taken into consideration. All descriptive statistics (mean, calculation of standard deviation and standard error of the mean values, ANOVA) were computed by using statistical software (SPSS 15.0, SPSS Inc., Chicago, USA).

Table 1 *In vitro* susceptibility of all 105 *Prototheca zopfii* strains to the different polyene drugs

Number of *P. zopfii* strains		MIC (μg/ml)			
		AMB	NYST	PIM	FIL
105	MIC_{50}	4	8	4	8
	MIC_{90}	4	16	8	16
	Range	0.5–32	2–32	1–32	4–32

AMB, amphotericin B; NYST, nystatin; PIM, pimaricin; FIL, filipin.

Results

The *in vitro* susceptibilities of the *P. zopfii* strains towards the different polyenes are summarized in Table 1. On the whole, amphotericin B and pimaricin exhibited the highest activity with the MIC_{90} of amphotericin B and pimaricin being 4 and 8 μg/ml, respectively, whereas the MIC_{90} of nystatin and filipin were from two to four times higher (Table 1). Comparison of the MIC values of the four polyenes with a non-parametric Wilcoxon test showed a high significance ($P<0.001$). Two strains were not susceptible to the highest concentration (32 μg/ml) of any polyene tested (Table 1). Individual MICs obtained by using the reference strain (*P. zopfii* NRRL Y-6868) gave results comparable with those reported in Table 1 (2 μg/ml for amphotericin B and nystatin and 4 μg/ml for pimaricin and filipin). In addition, MICs obtained in studies of the QC yeast strains for amphotericin B fell within the expected range (1 μg/ml for *C. parapsilosis* ATCC 22019 and 2 μg/ml for *Candida krusei* ATCC 6258, after 48 h) [21]. Similarly, nystatin, pimaricin and filipin were effective at concentrations from 1 to 2 μg/ml against the QC strains.

In order to verify the existence of different susceptibility patterns, the above data were reorganized by clustering the strains on the basis of their origin (Table 2). With the sole exception of nystatin susceptibility (which exhibited a P values $=0.051$, then close to statistical significance), no significant ($P<0.05$) differences in the polyene susceptibilities were noted among different strain clusters.

Discussion

The mechanism of action of polyene antibiotics is related to an increase in membrane permeability as the antibiotic binds to the sterols components (mainly ergosterol) and as a consequence modifies membrane functionality. Amphotericin B (produced by *Streptomyces nodosus*) is currently used in the treatment of severe systemic or cutaneous mycoses caused by many yeasts and filamentous fungi [22,23]. Nystatin (produced by *Streptomyces noursei*), active against yeasts

Table 2 *In vitro* susceptibility of all 105 *Prototheca zopfii* strains to the different polyene drugs by clustering the strains on the basis of their origin

Group (number of strains)		MIC (μg/ml)			
		AMB	NYST	PIM	FIL
1 (22)	MIC_{50}	2	4	2	8
	MIC_{90}	4	8	4	8
	Range	0.5–32	2–32	1–32	4–32
2 (23)	MIC_{50}	4	8	4	8
	MIC_{90}	4	16	4	16
	Range	1–4	8–16	4–8	8–16
3 (23)	MIC_{50}	2	8	2	8
	MIC_{90}	4	8	8	8
	Range	0.5–8	2–8	1–8	4–8
4 (23)	MIC_{50}	4	8	4	8
	MIC_{90}	4	16	8	16
	Range	4–8	8–16	4–8	8–16
5 (14)	MIC_{50}	4	8	8	8
	MIC_{90}	4	8	8	8
	Range	1–4	2–8	4–8	4–8

AMB, amphotericin B; NYST, nystatin; PIM, pimaricin; FIL, filipin.
The geographical origin of groups 1–5 is reported in the text.

(e.g., *Candida* spp.), is employed in the treatment of cutaneous and mucosal candidiases or, in combination with other antibiotics, to suppress their overgrowth in the gastrointestinal flora [22,23]. Pimaricin (also labelled as natamycin and produced by *Streptomyces natalensis*), which is active against yeasts (*Candida* spp.), filamentous fungi (*Fusarium* spp.) and protozoa (*Trichomonas vaginalis*), is employed for the localized treatment of candidiases, fungal keratites and for the control of vaginal trichomoniases [22,23]. Filipin is a complex of polyene antibiotics produced by *Streptomyces filipinensis*. This drug shows antimycotic activity against yeasts, but is considered too toxic for therapeutic applications [24–26].

To the author's knowledge, this is the first study reporting the *in vitro* susceptibility of a large set of *P. zopfii* strains towards polyene antibiotics. In consideration of the activity target of these drugs, it would appear that the different *in vitro* susceptibilities observed could be due to variable ergosterol content of the cell membranes of the different strain employed in the studies. This could also explain the resistance of some strains to these antifungals. In some cases, the MICs of both amphotericin B and nystatin were several orders of magnitude higher than those reported in previous studies. This may be mainly the result of different testing methods employed in previous investigations [15,20]. The results of the present study were obtained with RPMI 1640, a susceptibility testing medium for yeasts suggested by CLSI guidelines [21]. In contrast, Segal *et al*. [15] and Marques *et al*. [20] tested the antimycotic activity of both amphotericin B and nystatin on M-20 Antibiotic medium and Brain Heart Infusion broth, respectively. In addition, these two investigations provided only an incomplete overview of *P. zopfii* polyene susceptibility patterns because both involved a very limited number of test isolates [15,20]. On the basis of the results from the present study, it is possible to conclude that the four polyenes tested (in particular amphotericin B and pimaricin) exhibit variable *in vitro* activity towards *P. zopfii* strains. Finally, serious considerations should be given to the finding that some strains were resistant to 32 μg/ml of all polyenes, especially since this is in agreement with previously published preliminary data from studies involving *P. zopfii* susceptibility to amphotericin B [14].

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