Short Communication: In Vitro Antimicrobial Susceptibility of *Prototheca wickerhamii* and *Prototheca zopfii* Isolated from Bovine Mastitis

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ABSTRACT

Bovine mastitis caused by *Prototheca* spp. can assume high significance because of economic losses and the potential risk to public health. Studies on the susceptibility of *Prototheca* spp. to antimicrobials have demonstrated its high level of resistance. We report the susceptibility of bovine isolates of *Prototheca wickerhamii* and *Prototheca zopfii* to amphotericin B and nystatin, 2 antifungal agents commonly used in the control of protothecosis, and discuss the results. After subculture, minimum inhibitory concentrations of both antifungal drugs were determined using macrodilution and agar diffusion methods. The inoculum concentration was standardized by determination of colony-forming units per milliliter. Nystatin showed more efficacy than amphotericin B in inhibiting *P. wickerhamii* growth. In contrast, growth inhibition of *P. zopfii* was similar for both antifungal agents. This study demonstrates different in vitro susceptibility patterns of *P. wickerhamii* and *P. zopfii*, reinforcing the necessity for more investigation into drugs that can be used with clinical efficacy.

Key words: bovine mastitis, antimicrobial susceptibility, *Prototheca*

Bovine mastitis in both its clinical and subclinical forms continues to result in substantial loss of milk production and reduced milk quality. The use of antibiotics and the implementation of effective control programs for contagious pathogens are contributing to the predominance of environmental mastitis pathogens, especially in well-managed herds (Cattell et al., 2001). In many instances, the indiscriminate use of antimicrobial agents in the treatment of mastitis in dairy cattle is increasing the selection pressure toward antimicrobial resistance. A single strain may predominate because of antimicrobial resistance, host adaptation, or other factors (Lehtolainen et al., 2003). Bovine mastitis

caused by algae of the genus *Prototheca* is gaining economic and public health importance, and its increasing incidence may be explained by these mechanisms (Benites et al., 1999; Melville et al., 2002; Roesler and Hensel, 2003).

Members of the genus *Prototheca* are unicellular algae related to the green algae of the genus *Chlorella*, but without chlorophyll (Pore, 1998; Hollingsworth, 2000; DiPersio, 2001; Rodriguez, 2003). They are resistant and ubiquitous and can be isolated from a great variety of environmental sources (Melville et al., 1999; Bexiga et al., 2003; Zhao et al., 2004). Only 2 species are pathogenic, *Prototheca wickerhamii* and *Prototheca zopfii* (Pore, 1998; Malinowski et al., 2002; Melville et al., 2002). Protothecosis in cattle is generally associated with *P. zopfii*, but recently, and for the first time, we identified one case of bovine mastitis caused by *P. wickerhamii*. In humans, protothecosis is almost exclusively associated with *P. wickerhamii* (DiPersio, 2001; Roesler et al., 2001; Roesler and Hensel, 2003; Linares et al., 2005). *Prototheca* as a cause of mastitis in dairy cows depends on predisposing factors such as poor environmental conditions and insufficient milking hygiene (Roesler et al., 2001; Roesler and Hensel, 2003). Infection by these algae causes acute to chronic granulomatous mastitis, leading to reduced milk production and atresia of the udder. Because of its capacity to infect and survive in macrophages and to invade the mammary tissue, they are responsible for a persistent infection with intermittent shedding (Costa et al., 1997; Benites et al., 1999; Roesler et al., 2001; Roesler and Hensel, 2003), and transmission may be by direct contact (Costa et al., 1997; Roesler et al., 2001; Bexiga et al., 2003). These algae do not respond to routine mastitis therapy, and the only control method to date has been elimination of the infected animals (Costa et al., 1997; Benites et al., 1999; Melville et al., 1999; Roesler et al., 2001).

Reports of in vivo and in vitro susceptibility studies of these algae to the usual mastitis antimicrobial agents indicate that they are resistant in the majority of cases (Melville et al., 2002; Bexiga et al., 2003; Zhao et al., 2004). However, Segal et al. (1976) found in vitro susceptibility of *P. zopfii* isolates to 2 polyenic antifungal

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agents commonly used in human protothecosis therapy. These findings led us to test the in vitro susceptibility of both *P. zopfii* and *P. wickerhamii* obtained from bovine mastitis to these antifungal drugs, with the objective of studying their antimicrobial resistance patterns.

For this study, one *P. wickerhamii* and 8 *P. zopfii* isolates previously obtained from clinical and subclinical bovine mastitis were revived by subculture on Sabouraud 4% dextrose agar (Merck, Darmstadt, Germany) and incubated for 48 to 72 h at 37°C. Speciation was performed using routine culture, macro- and microscopic morphological characterization, and API 20C AUX (bioMérieux, Marcy l'Etoile, France) methods (Pore, 1998), and confirmed by PCR (G. Thompson, unpublished data). Although there are no guidelines or interpretative criteria specific for *Prototheca* spp., the National Committee for Clinical Laboratory Standards (NCCLS, Clinical and Laboratory Standards Institute) M27-A2 guidelines for preparation of media and inoculum were followed (NCCLS, 2002). For MIC determination, macrodilution in brain heart infusion broth (Merck) and agar diffusion in Mueller-Hinton agar acc. NCCLS (Merck) were used. The selected antimicrobials were amphotericin B (AMB) 250 µg/mL (Sigma-Aldrich, Sintra, Portugal) and nystatin (NYT) 5000 IU/mL (manufactured by Lemos Pharmacy, Oporto, Portugal). Both antifungal drugs were diluted (1:2) in brain heart infusion broth with a concentration range from 0.00763 to 125 µg/mL for AMB and from 0.0305 to 500 µg/mL for NYT. The colony forming units of each isolate were between 0.74×10^6 and 9.1×10^6 cfu/mL for *P. zopfii* and were 2.8×10^5 cfu/mL for *P. wickerhamii*. A volume of 20 µL from each concentration of each *Prototheca* isolate was then inoculated into each dilution of the antifungal drug. Following an incubation period of 48 to 72 h at 37°C, 100 µL of each dilution was spread on Sabouraud 4% dextrose agar and incubated under the same conditions to confirm growth inhibition. In the macrodilution method, the MIC corresponds to the last tube without turbidity. To determine the growth inhibition diameter, the agar diffusion method was used. Each *Prototheca* spp. inoculum was streaked on Mueller-Hinton agar plates and blank susceptibility disks were placed on these plates after being submerged in the drug dilution tubes. The plates were incubated for 48 to 72 h at 37°C and the inhibition diameters were measured. The MIC obtained for *P. zopfii* and *P. wickerhamii* are summarized in Table 1. For *P. zopfii*, the AMB and NYT minimum growth inhibition was measured at 10 and 8 mm in diameter, respectively, and for *P. wickerhamii*, this value corresponded to a growth inhibition diameter of 22 and 10 mm for AMB and NYT, respectively.

Table 1. Minimum inhibitory concentration for *Prototheca zopfii* and *Prototheca wickerhamii* isolated from bovine mammary glands

Antifungal agent	MIC (µg/mL)		
	50% ¹	90% ²	Range
Against <i>P. zopfii</i>			
AMB ³	0.488	0.976	0.244 to 0.976
NYT ⁴	0.488	1.953	0.122 to 1.953
MIC (µg/mL)			
Against <i>P. wickerhamii</i>			
AMB		7.812	
NYT		1.953	

¹Lowest concentration inhibiting 50% of the isolates tested.

²Lowest concentration inhibiting 90% of the isolates tested.

³Amphotericin B (Sigma-Aldrich, Sintra, Portugal).

⁴Nystatin (manufactured by Lemos Pharmacy, Oporto, Portugal).

This is the first study to evaluate the susceptibility of a bovine isolate of *P. wickerhamii* to antifungal drugs. Nystatin showed more efficacy than AMB in inhibiting growth of this alga. However, more in vitro and in vivo studies are essential to substantiate these results, because only one isolate was tested owing to its rare involvement in bovine mastitis. On the other hand, *P. zopfii* growth was similarly inhibited by both antifungal agents, even though AMB had a higher MIC₉₀ than NYT (Table 1). In a study by Malinowski et al. (2002), all *P. zopfii* strains obtained from the udders of cows were also sensitive to NYT at 100 IU, which is in agreement with our results. In another study (Buzzini et al., 2004), NYT was found to inhibit the growth of 58% of the *Prototheca* strains, compared with 33% growth inhibition for AMB, both at 50 µg/mL. Bovine mastitis by *P. wickerhamii* is a rare event and, although based in one isolate, our results suggest that growth of this *Prototheca* spp. is less affected than *P. zopfii* by both antifungals studied, as indicated by a systematic smaller diameter of the inhibition zone. These results demonstrate the need for a greater number of samples to achieve more conclusive results.

There are still no drugs with proven clinical efficacy against protothecal bovine mastitis (Bexiga et al., 2003). Although our results indicate some sensitivity to these antifungal drugs in vitro, this does not guarantee that the drugs can be used for therapy against infections with these pathogens in cows. In other in vitro susceptibility studies, these algae have shown variable but low sensitivity to some antibiotics (Bexiga et al., 2003; Zhao et al., 2004). Amphotericin B is widely used in human medicine to treat protothecal infections (Di-Persio, 2001; Rodriguez, 2003; Linares et al., 2005) and demonstrates good activity, with MIC ranging from 0.09 to 3.12 µg/mL (Segal et al., 1976). Recently, Voriconazole demonstrated greater activity (MIC₉₀ = 0.5 µg/

mL) than AMB (MIC₉₀ = 1 µg/mL) against all *P. wickerhamii* strains isolated from humans (Linares et al., 2005), and testing on bovine *Prototheca* sp. isolates is warranted. The different patterns of susceptibility to AMB and NYT shown by *P. wickerhamii* and *P. zopfii* in this study imply the need for further investigation into drugs that can be used with similar efficacy and, at the same time, be approved for use for intramammary or intramuscular administration in cattle. Mastitis caused by *P. wickerhamii* is very rare, and the pattern of susceptibility seen here needs to be confirmed with further studies.

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