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Susceptibility of yeast isolates from cattle with otitis to aqueous solution of povidone iodine and to alcohol-ether solution

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Lipid-dependent *Malassezia* species, *Candida* spp. and *Rhodotorula mucilaginosa* have been associated with bovine parasitic otitis. This paper evaluated the susceptibility of 63 yeast isolates from cattle with otitis to a povidone iodine aqueous solution (1% and 0.5% v/v) and to an alcohol-ether solution (1:1 v/v). The effectiveness of these antiseptics was assessed using the European suspension test. Products achieving equal to or greater than 5-log reduction in numbers of the challenge organism after 5 min contact are considered to have as acceptable microbicidal effect (ME). The two antiseptic solutions achieved ME greater than 5, when tested at 1 and 5 min contact time, against the majority of yeast strains. The exceptions were alcohol and ether solution against two *Candida tropicalis* strains. Urea broth macrodilution method was used to determine the minimum inhibitory concentration (MIC), defined as the lowest concentration that resulted in a visually negative urease test or, in the case of *Candida* spp., turbidity inhibition when compared with that produced by the growth control. Analysis of the results for all 63 isolates showed *Malassezia sympodialis* and *Rhodotorula mucilaginosa* to be more susceptible to povidone iodine and *Malassezia furfur* strains to be less susceptible. *Malassezia sympodialis* was significantly more susceptible to alcohol-ether solution than other species. This study showed the *in vitro* efficacy of alcohol-ether solution and povidone iodine and proposes the need for clinical evaluation of the topical treatment and control of bovine otitis with these antiseptics and their effects on the ear microbiota and the ear canal.

Keywords antiseptics, bovine otitis, yeast.

Introduction

Otitis in cattle has a significant impact in tropical and subtropical regions and the etiological agents are predominantly rhabditiform nematodes and mites of the genus *Raillietia*. In advanced clinical cases there can be irreversible and fatal neural lesions [1]. Healing can be compromised by the participation of secondary agents which occur in many of the cattle with chronic parasitic otitis, especially when this disease is associated with otitis media, internal otitis, dysfunction or paralysis of the facial and vestibulocochlear nerves and meningitis [2–4].

Treatment of bovine parasitic otitis is often undertaken using a solution of ethyl alcohol and ethyl ether in a proportion of 1:1 (v/v). The therapeutic action of this solution, besides having a direct effect on the pathogens, has also been attributed to its effect on cerumen. The alcohol has a dehydrating effect and the ether is a lipid solvent. Although this treatment is 100% effective against nematodes, re-infection is common [5]. The effect of this treatment against bacteria and yeast has yet to be established.

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Lipid-dependent *Malassezia* species have been associated with bovine parasitic otitis. *Malassezia globosa*, *M. sympodialis*, *M. furfur* and *M. slooffiae* were the most frequently identified species among isolates obtained from cattle with otitis [6]. In a mycological study, the genus *Malassezia* was the fungus most prevalent from Gyr cows with parasitic otitis. The cultures also revealed the growth of *Candida* spp., *Rhodotorula mucilaginosa* and *Aspergillus* spp. [7].

The activity of ethyl alcohol and ethyl ether solution and povidone iodine and other antiseptics is not known in yeast of the genus *Malassezia* or other yeasts isolated from cattle with otitis. The purpose of this study was to evaluate the susceptibility of yeast isolated from cattle with otitis to povidone iodine and alcohol-ether solution.

Materials and methods

Strains

The strains were isolated from 147 Gyr cows with otitis from the central region of Minas Gerais state, Brazil, during previous studies [7,8].

The yeasts of the genus *Malassezia* were identified using the following criteria: morphological characteristics, growth on Dixon medium at different temperatures (32, 37 and 40°C), growth on Sabouraud's medium agar supplemented with Tween 20, 40, 80 or cremophor EL, esculin and catalysis screening and synthesis of flurochromes and pigments [9–11]. The genus *Candida* and *Rhodotorula* were identified based on micromorphological studies and biochemical tests according to standard methods [12]. The yeasts were identified through the use of the keys in Kurtzman and Fell [13].

Susceptibility to antiseptics was evaluated in 15 isolates of *M. furfur*, 12 *M. sympodialis*, 12 *M. slooffiae*, 12 *Candida* spp., and 12 *Rhodotorula mucilaginosa*. The *Candida* strains corresponded to five *C. albicans*, one *C. castelli*, one *C. guilliermondii*, one *C. krusei*, one *C. lusitaniae*, one *C. parapsilosis*, one *C. pararugosa* and one *C. tropicalis*. The standard strains CBS-1878 (*M. furfur*), CBS-1879 (*M. pachydermatis*), ATCC-A 18804 (*C. albicans*), ATCC-T 750 (*C. tropicalis*) and ATCC-G 2001 (*C. glabrata*) were simultaneously tested.

The media used for the maintenance of test yeasts and for viable counts was modified Dixon agar at 32°C for *Malassezia* strains and Sabouraud dextrose agar (Difco LTDA, Sparks, MD, USA) at 28°C for *Candida* and *Rhodotorula* strains. These isolates were subcultured every week more than three times before use. Test suspensions were prepared from 24 h subcultures of *Candida* and *Rhodotorula* strains and 72 h subcultures of *Malassezia* strains. The colonies grown were collected by scraping and suspending in sterile distilled water (pH 7.0) with a glass homogenizer.

Antiseptics

The yeasts were tested for their susceptibility to ethyl alcohol and ethyl ether (Labsynth, São Paulo, Brazil) in a proportion of 1:1 (v/v) and povidone iodine aqueous solution 1% and 0.5% v/v (Biosintética, São Paulo, Brazil). In preliminary studies was evaluated the susceptibility to boric acid (Labsynth) 3% v/v.

Quantitative suspension test

The yeast suspension was adjusted to an absorbance (optical density at 660 nm of 1.0) corresponding to $1-5\times10^7$ cells/ml for *Malassezia* strains and $1-5\times10^8$ cells/ml for *Candida* and *Rhodotorula* strains. The suspensions were maintained at 20°C and used within 2 h.

Tests were carried out according to the European Standard EN 12761276 quantitative suspension test for the evaluation of chemical disinfectants and antiseptics [14,15]. Before starting the test, all reagents were equilibrated to 20°C in a water-bath. The antiseptic test solution (9.9 ml) was added to 100 µl test yeast suspension, mixed by vortexing and returned to the 20°C water-bath. One and 5 min after the addition of the test yeast suspension, 1 ml was removed and added to 9 ml of sterile distilled water [14,15]. Further decimal dilutions were made and 1ml inoculated onto plates with the appropriate media (modified Dixon agar or Dextrose Sabouraud agar) at 45°C. The plates were incubated at 32°C for 72 h (yeast of the genus *Malassezia*) or 28°C for 24 h (yeast of the genus *Candida* and *Rhodotorula*). In control tests, the 100 µl test yeast suspension was added to 9.9 ml sterile distilled water and decimally diluted in the same way.

The microbicidal effect (ME) was calculated by subtracting the log number of colony-forming units (c.f.u.) per ml after action of the antiseptic from the log c.f.u./ml of the control test. Products achieving equal to, or greater than a 5-log reduction in number of the challenge yeast after a 5-min contact time were considered to have acceptable ME.

Determination of minimum inhibitory concentration

The urea broth macrodilution method using the modified protocol, described by Nakamura *et al*. [16], was used to determine the minimum inhibitory con-

centration (MIC) of povidone iodine and alcohol-ether 1:1 v/v solution. These antiseptics were diluted in sterile distilled water (pH 7.0) and the highest test concentration was 125 µl/ml alcohol and 125 µl/ml ether, and 1.25 mg/ml of povidone iodine.

The yeast suspensions were adjusted to an absorbance (optical density at 660 nm, corresponding to $1-5\times106$ cells/ml). The urea broth macrodilution method of susceptibility testing was performed in a sterile test tube with 500 µl Christensen's urea broth modified to grow lipophilic yeast by the addition of Tween 40 (0.5%) and Tween 80 (0.1%) [16], yeast suspension (250 µl) and the test antiseptic dilution. The tests were conducted in triplicate.

The test tubes were incubated at 32°C for 48 h for yeasts of the genus *Malassezia* or at 28°C for 24 h for *Candida* and *Rhodotorula* strains. Phenol red used as an indicator was very sensitive to fungal cell activity. All yeasts of the genus *Malassezia* and *Rhodotorula* were positive for urease activity when modified Christensen's urea broth was used. The *Candida* species were urease negative. MIC were defined as the lowest concentration that resulted in a visual negative urease or, in case of *Candida* spp., turbidity inhibition when compared with that produced by the growth control. These results were statistically analysed with nonparametric Kruskal–Wallis test.

Results

In preliminary studies an ME <1 to the quantitative suspension test of boric acid 3% v/v added to yeast suspensions of two strains of each species of test fungi was observed [E.R.D., unpublished results]. The quantitative evaluation of the susceptibilities of the test yeast to ethyl alcohol and ethyl ether solution (1:1 v/v), povidone iodine (1% v/v) and povidone iodine (0.5% v/v) are shown in Table 1. Ethyl alcohol/ethyl ether solution, as well as 0.5% and 1.0% povidone iodine, when examined after 1 and 5 minutes of contact with the test isolates, achieved at least an ME of 5 (5 log reduction). The exceptions were alcohol and ether solution against VG Rc Oiana and ATCC-T 750 (*C. tropicalis* strains). Reduction in activity of this solution was more pronounced when contact time was reduced from 5 to 1 min.

The determination of MIC of povidone iodine and alcohol ether solution to the test yeast, using the urea broth macrodilution method, is shown in Table 2. *Malassezia sympodialis* and *Rhodotorula mucilaginosa* were the yeasts more susceptible to povidone iodine. *Malassezia furfur* strains were less susceptible than other test yeasts ($P<0.05$). In urea broth macrodilution of alcohol ether solution 1/1 v:v the *M. sympodialis* was significantly more susceptible than other test species ($P<0.05$).

Discussion

Boric acid has been used for topical treatment of some fungal infections. In our preliminary studies we observed an ME <1 when boric acid was added to yeast suspensions (3% v/v). A study of the susceptibility of *Candida* spp. to boric acid showed that *C. glabrata* was more inhibited *in vitro* than *C. albicans* [17]. This solution has been added to external otitis in cattle in some Brazilian states to control bovine otitis [5]. We observed the significant resistance of yeast from cattle with otitis to this antiseptic and its efficacy in the treatment of bovine otitis with fungal participation is not indicated.

Ethyl alcohol/ethyl ether solution, in quantitative suspension tests, achieved the pass criterion of at least a 5 log reduction (ME = 5) for the majority of yeasts strains studied. Both substances are important in the cleaning of the external ear and reduction of the nutrients available to the yeasts. Little is known about the specific mode of action of alcohol on yeast cells. It is generally believed that it causes membrane damage and rapid denaturation of proteins, with subsequent interference with metabolism and cell lyses [18].

The lower susceptibility of *C. tropicalis* strains suggests that the use of this alcohol-ether solution could select this species in mixed infections of bovine ear. Ethanol has been reported to induce mycelial development in *C. tropicalis*. Comparison of the *in vitro* activity of antiseptics to isolates of *Candida* spp. has shown that *C. tropicalis* is resistant to the antiseptics Hibiclens (chlorhexidine gluconate 4%) and Clinidine (povidone iodine 1%) [19]. *Candida tropicalis* also shows a high affinity and tolerance towards phenol. Some studies have shown that *C. tropicalis* has a high ability to degrade phenol. This species can utilize phenol in concentrations up to 2.5 per litre as a sole carbon and energy source [20,21].

The test of urea macrodilution of ethyl alcohol/ethyl ether showed that it should not be diluted because the majority of test yeasts have MIC higher than 250 µl/ml alcohol with 250 µl/ml ether solution, as shown in Table 2. *M. sympodialis* was significantly more susceptible than other *Malassezia* species. In future, research analysis of the differences in lipid composition of the yeast present in bovine otitis may explain the variations observed in their susceptibility to ethyl alcohol/ethyl ether solutions.

Table 1 Microbicidal effect (ME) of ethyl alcohol and ethyl ether 1:1 (v/v) solution, 1% and 0.5% v/v povidone iodine against yeast from cattle with otitis after 1 and 5-min contact time in quantitative suspension test

Test yeast	No. of strains	Alcohol and ether 1:1 v/v		Povidone iodine 1% v/v		Povidone iodine 0.5% v/v	
		1 min	5 min	1 min	5 min	1 min	5 min
M. sympodialis	12	6.89–7.46	7.06–7.46	6.89–7.46	7.06–7.46	6.89–7.46	7.06–7.46
M. slooffiea	12	6.8–7.59	6.94–7.59	6.8–7.59	6.94–7.59	6.8–7.59	6.94–7.59
M. fufur	15	6.94–7.68	6.79–7.69	6.94–7.68	6.79–7.69	6.94–7.68	6.79–7.69
CBS1878	1	7.28	7.12	7.28	7.12	7.28	7.12
CBS 1879	1	7.12	7.11	7.12	7.11	7.12	7.11
Candida spp.	11	7.78–8.69	8.02–8.65	7.78–8.69	8.02–8.65	7.78–8.69	8.02–8.65
VG Rc Oiana†	1	3.36*	3.6*	8.54	8.53	8.54	8.53
ATCC–T 750†	1	3.57*	4.48*	8.18	8.02	8.18	8.02
ATCC–A 18804‡	1	8.57	8.45	8.57	8.45	8.57	8.45
ATCC–G 2001§	1	8.45	8.52	8.45	8.52	8.45	8.52
R. mucilaginosa	12	8.31–8.59	8.33–8.6	8.31–8.59	8.33–8.6	8.31–8.59	8.33–8.6

*Microbicidal effect <5; †*Candida tropicalis* strains; ‡*Candida albicans*; §*Candida glabrata*.

The effect of polidocanol (dodecypoly-ethyleneglycolether) was evaluated in a study of different *Malassezia* species. The strains of *M. sympodialis* were significantly more sensitive and had MIC one-tenth of those found for *M. furfur* [22].

The quantitative suspension test to 1% v/v povidone iodine and 0.5% v/v povidone iodine showed the ME greater than 5 against all yeast strains. Iodine rapidly penetrates into microorganisms and attacks key groups of proteins (in particular the free-sulfur amino acids cysteine and methionine), nucleotides and fatty acids, which culminates in cell death [18].

In the determination of MIC the lowest values observed suggested that these antiseptic solutions could be more diluted. In the topical treatment of bovine otitis, the 1.0 or 0.5% dilution of povidone iodine will be probably more efficient than other lower concentrations because of the significant presence of organic matter in external ears of cattle.

In this study a higher susceptibility of *M. sympodialis* and *Rhodotorula mucilaginosa* strains to povidone iodine was observed. *Malassezia furfur* strains were less susceptible than other test yeasts. These differences may be explained by the variation of contact which these species have had with povidone iodine which have selected strains more tolerant to this agent.

The cerumen will probably have effects on the microbicidal efficacy of the two antiseptic solutions, as would many other physical and biological environmental factors found in the bovine ear canal. There are no data on the effects of these antiseptics in the bovine ear canal and there are very few studies into the pathophysiology of bovine otitis. When the tympanic membrane is ruptured or in neural lesions, the topical treatment with these products is contraindicated.

This study showed the *in vitro* efficacy of alcohol-ether solution and povidone iodine to reduce the yeast population from bovine otitis. Futures studies will

Table 2 The minimum inhibitory concentrations (MIC) for antiseptic solutions to reduce yeast from cattle with otitis populations over 6-log10 using the urea broth macrodilution method

Test yeast	No. of strains	Alcohol and ether 1/1 v:v			Povidone iodine		
		Mean µl/ml	%	Range µl/ml	Mean mg/ml	%	Range mg/ml
M. sympodialis	12	125*	12.5	125– >250	0.039*	0.0039	0.039–0.625
M. slooffiea	12	>250	>25	250– >250	0.625	0.0625	0.039–1.25
M. fufur	15	>250	>25	250– >250	1.25*	0.125	0.078–1.25
CBS 1878 (*M. furfur*)	1	>250	>25	>250	0.312	0.0312	0.312
CBS 1879(M. pachydermatis)	1	>250	>25	>250	0.625	0.0625	0.625
Candida spp.	11	>250	>25	250– >250	0.156	0.0156	0.156–0.625
VG Rc Oiana†	1	>250	>25	>250	0.312	0.0312	0.312
ATCC–T 750†	1	>250	>25	>250	0.312	0.0312	0.312
ATCC–A 18804‡	1	>250	>25	>250	0.312	0.0312	0.312
ATCC–G 2001§	1	>250	>25	>250	0.625	0.0625	0.625
R. mucilaginosa	12	>250	>25	>250	0.039*	0.0039	0.039

*P <0.05, Kruskal–Wallis test; †*Candida tropicalis* strains; ‡*Candida albicans*; §*Candida glabrata*.

be needed to clinically evaluate the efficacy of alcohol-ether solution and povidone iodine solution for the topical treatment and control of bovine otitis and their effect on the ear microbiota and ear bovine tissues.

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