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Natamycin as a Selective Antifungal Agent in Media for Growth of *Legionella* spp.

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The growth of 18 different *Legionella* sp. strains and 76 different yeast isolates was tested on buffered charcoal yeast extract medium supplemented with α-ketoglutarate (BCYEα medium) and with natamycin, an antifungal agent. Bacterial growth was no different on BCYEα medium made with or without natamycin, whereas complete inhibition of yeasts occurred in BCYEα medium containing 200 to 500 μg of natamycin per ml. Selective BCYEα media made with natamycin rather than anisomycin had no (formulation with vancomycin, polymyxin B, and agar) or little (formulation with cefamandole, polymyxin B, and agar) inhibitory effect on the growth of 14 different *Legionella* sp. bacteria. Natamycin is an inexpensive alternative to anisomycin in the formulation of selective BCYEα media.

Yeast frequently overgrow *Legionella pneumophila* on nonselective buffered charcoal yeast extract medium supplemented with α-ketoglutarate (BCYEα medium) plated with clinical samples. This led to the use of the antifungal agent anisomycin in selective BCYEα medium, which generally works well in inhibiting yeasts and, less so, molds (2). However, anisomycin is extraordinarily expensive ($1,123/5 g; Organon Technica Co., Durham, N.C.), adding about $0.45 to the materials cost of a single selective plate. In addition, anisomycin must first be solubilized by acidification before addition to the medium, which is a time-consuming and somewhat complex process. Natamycin is an antifungal agent widely used in the cheese industry to retard mold formation, as an ophthalmologic antifungal agent, and as an antifungal agent in selective media used to culture environmental specimens (1, 6, 8). In addition, some have used it in selective BCYEα media, although no data exist as to its utility in such media (10). Natamycin is not known to be active against bacteria, because it is a polyene antimicrobial agent which inhibits sterol synthesis. The drug is relatively inexpensive ($860/kg) and has a very long shelf-life (1). We report on the ability of natamycin to inhibit yeast plated onto natamycin-containing BCYEα medium, as well as its lack of an effect on the growth of several different *Legionella* species.

BCYEα medium was prepared as described previously with morpholinepropanesulfonic acid (MOPS) buffer (4). Two different selective BCYEα-based media were also tested: BCYEα medium with cefamandole and polymyxin B (BMP) and BCYEα medium with vancomycin and polymyxin B (YE) (3). Two different natamycin preparations were tested, as were two different methods of adding the drug to the medium. Pure medical-grade natamycin was provided by Alcon Laboratories, Inc. (Ft. Worth, Tex.), and commercially available food-grade natamycin (Delvocid Instant) was obtained from Gist-Brocades (Menomonee Falls, Wis.). Delvocid Instant contains (by weight) 50% natamycin and 50% lactose and costs $86.50/100 g. Neither natamycin preparation is sterile or soluble at high concentrations in aqueous or organic solvents (1, 7). Natamycin was suspended (≈30 mg/ml) in 0.1 N NaOH and was then added to cooled (50°C) molten autoclaved BCYEα medium. Alternatively, the drug was added in powder form to BCYEα medium before autoclaving to determine if the solubilization step could be omitted.

The media were tested by using frozen homogenized suspensions of lungs from guinea pigs with *L. pneumophila* serogroup 1 (strain F889) pneumonia (5) and up to 17 other previously frozen *Legionella* species strain (Table 1). The thawed frozen lung homogenate was plated directly without passage onto plates, and the other isolates were first passed twice on BCYEα medium before use. In experiments that studied selective media, the growth of some strains was so poor on the control selective media that comparisons could not be performed for all 18 strains. In addition, 58 isolates of freshly isolated yeasts from clinical specimens were tested; these were passed once on Sabouraud dextrose medium (Emmons modification) before use (Becton Dickinson Microbiology Systems, Cockeysville, Md.). The yeasts were identified as *Candida albicans* (25 isolates) on the basis of a positive germ tube assay, as *Cryptococcus neoformans* (3 isolates) on the basis of standard methods (11), or as yeasts that were neither *Candida albicans* nor *Cryptococcus neoformans* (30 isolates) on the basis of negative germ tube and urease tests (11). For the *Legionella* species strains, serial dilutions of suspensions approximating the turbidity of a half McFarland barium sulfate standard were quantitatively plated onto duplicate plates. For the yeasts, approximately 0.003 ml of a suspension approximating the turbidity of a number 2 McFarland standard was spot inoculated onto duplicate plates by use of either a Steers Foltz Graves inoculator (Chester Machine Shop, Chester, Pa.) or a micropipette. Incubation of all plates was done at 35°C in humidified air. The total number of colonies present on four culture plates (two dilutions in duplicate), which is referred to as a "plate set," was used for the quantification of bacterial colonies growing on the test media.

Standard descriptive statistics were calculated from the colony counts for each medium type, and the performance of different medium types were compared by using either repeated-measures one-way or two-way analysis of variance, paired Student's *t* test, or chi-square analysis. In all cases, two-tailed *P* values were determined (InStat version 2.02; GraphPad Software, San Diego, Calif.). Two-way analysis of variance calcu-

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TABLE 1. Identities of the 18 clinical *Legionella* isolates tested

Strain	*Legionella* species	Serogroup	Name
F237	*L. pneumophila*	1	
F624	*L. pneumophila*	4	Los Angeles 1
F736	*L. pneumophila*	1	
F774	*L. micdadei*	1	
F850	*L. longbeachae*	1	
F889	*L. pneumophila*	1	
F1344	*L. pneumophila*	1	
F1380	*L. pneumophila*	1	
F1478	*L. dumoffii*	1	TEX-KL
F1527	*L. pneumophila*	1	Knoxville
F1548	*L. longbeachae*	2	
F1717	*L. dumoffii*	1	
F1807	*L. pneumophila*	6	
F1821	*L. pneumophila*	1	
F1864	*L. pneumophila*	9	
F2111	*L. pneumophila*	1	
F2127	*L. pneumophila*	1	Philadelphia 1

lations were calculated by using SAS software (SAS Institute, Cary, N.C.). Unless otherwise stated, data presented in the form x (y to z) represent the mean and 95% confidence intervals.

Natamycin added to autoclaved BCYEα medium completely inhibited the growth of 56 of 58 (97%) yeast isolates (Table 2). Lower concentrations of natamycin were much less effective.

Autoclaving partially inactivated natamycin. Eleven of 18 yeast isolates different from the ones tested previously (9 *C. albicans* isolates and 9 yeast isolates other than *C. albicans* or *C. neoformans*) grew after 1 day of incubation on BCYEα medium to which natamycin (200 μg/ml) had been added before autoclaving. In contrast, there was complete inhibition, for at least 5 days, of all 18 yeast isolates inoculated onto BCYEα media containing natamycin added either after autoclaving (200 μg/ml) or before autoclaving at a higher concentration (500 μg/ml).

Natamycin (200 μg/ml added after autoclaving) had no effect on the growth of 18 different *Legionella* species strains inoculated onto BCYEα medium. The average number of colonies per plate set was 184 (147 to 220) for BCYEα medium with or without added natamycin (P = 0.8; paired t test). In another, independent experiment, natamycin addition to BCYEα medium before or after autoclaving did not affect the growth of 13 *Legionella* species strains. Strains F1548, F1717, F1821, F2111, and F2127 were not tested because of a technical error, thus accounting for 13 rather than 18 strains tested. An average of 333 (251 to 416) colonies per plate set were detected on BCYEα medium not containing natamycin, whereas 345 (258 to 434), 339 (251 to 428), and 338 (251 to 426) colonies were detected on media containing 200, 500, or 200 μg of natamycin per ml added before, before, and after autoclaving of the media, respectively ($P = 0.48$ and $F = 0.85$ by repeated-measures one-way analysis of variance).

The addition of natamycin (500 μg/ml postautoclaving) to YE selective medium did not affect the growth of 18 *Legionella* sp. strains. An average of 247 (185 to 311) colonies per plate set were counted on YE medium made with or without natamycin ($P = 0.99$; paired t test).

In contrast to the lack of natamycin inhibition of *Legionella* spp. grown on BCYEα or YE medium, there was slight, but significant, inhibition by natamycin of some *Legionella* sp. strains grown on BMP medium. Four independent experiments were performed in which we studied the ability of BMP medium, made with and without natamycin (500 μg/ml preautoclaving), to support the growth of 14 different *Legionella* sp. strains. Four of the 18 strains used for testing other media grew poorly (F774, F850, F1548, and F2127) or not at all on BMP medium, and therefore, their growth could not be tested on BMP medium to which natamycin was added. These experiments showed that the addition of natamycin to BMP medium significantly inhibited the growth of several of the bacterial strains tested ($P = 0.02$ and $F = 6.2$ by repeated-measures two-way analysis of variance). The differences between the media varied across the bacterial strains tested. This was seen by a significant medium-by-strain interaction term ($F = 2.6$; $P < 0.001$). The average numbers of colonies counted per plate set were 201 (159 to 244) and 181 (138 to 223) for BMP alone and BMP with natamycin, respectively. The differences between the media appeared slight, even though they were statistically significant. A measure of this is that in only one of four of the independent trials was there a statistically significant difference between the two media (by the paired t test), although in three of the four trials, growth was always better on BMP medium without natamycin. Some strains grew consistently better on one medium than on the other one. For example, the mean numbers of colonies counted per plate set ($n = 4$ independent experiments) for strain F2195 were 289 (164 to 414) and 221 (139 to 304) for BMP without and with natamycin, respectively. Strain F1821 grew much better on BMP with natamycin than in its absence, with an average of 155 (6 to 305) and 32 (0.5 to 63) colonies per plate set ($n = 4$ independent experiments), respectively.

Because such a study has not previously been conducted with anisomycin, we performed one experiment that compared the growth of the same 14 *Legionella* sp. strains on BMP, BMP made with anisomycin (80 μg/ml), and BMP made with natamycin (500 μg/ml). The mean numbers of colonies counted per plate set were 156 (85 to 227), 158 (90 to 225), and 136 (84 to 188) for BMP alone, BMP with anisomycin, and BMP with natamycin, respectively. No significant differences between the media were detected in a repeated-measures analysis of variance test ($P = 0.16$; $F = 1.99$).

These results demonstrate no significant differences between the performances of BCYEα or YE media made with or without natamycin. The growth of some *Legionella* spp. was significantly decreased on BMP medium made with natamycin compared with that on BMP medium alone. It is unclear if the statistically significant difference that was detected is of practical importance for several reasons. One is that it appears to be strain dependent, with some bacterial strains actually having markedly enhanced growth in the presence of natamycin. Another is that the mean differences were relatively small. Finally, it is unclear if the quantitative differences detected in our

TABLE 2. Inhibition of yeasts by natamycin added to BCYEα medium

Natamycin concn (μg/ml)	No. (%) of yeasts completely inhibited after incubation for:		
	2 days	3 days	8 days
25	0 (0)	ND[a]	ND
50	22 (38)	ND	ND
100	40 (69)	ND	ND
200	56 (97)	15[b] (100)	14 (93)

[a] ND, not determined.

[b] Only 15 of the 56 strains completely inhibited at 2 days were tested and these strains were obtained by random selection.

studies would be reflected in qualitative differences in clinical specimens. Because one of the test inocula (strain F889) studied was *L. pneumophila*-infected lung tissue, these results are likely representative of the clinical performance of these media for *L. pneumophila.*

The mechanism of natamycin growth antagonism or enhancement with BMP medium, but not with YE or BCYEα medium, is unclear. It appears that the interaction must be with cefamandole, because this is present in BMP medium but not in YE medium. Cefamandole is known to inhibit the growth of several different *Legionella* spp., as well as some *L. pneumophila* strains (9). Perhaps strains that are borderline susceptible to cefamandole are made more so by the addition of natamycin.

We now use natamycin (500 mg/liter added before autoclaving), rather than anisomycin, in all of our selective media used for the culturing of both clinical and environmental specimens. Changing to natamycin has resulted in a major cost savings, with a materials cost reduction of $0.43 per plate. The ease of adding the drug to the medium before autoclaving results in about 15 min of time savings per batch of medium. Even though the commercial cost of anisomycin is undoubtedly lower than our costs, there would still be major cost savings if medium manufacturers switched to natamycin.

On the basis of cost considerations alone, there is no longer a role for the use of anisomycin in YE, and probably BMP, selective media for *Legionella* spp. We believe that it is cost-effective to substitute natamycin for anisomycin in all selective media used to grow *Legionella* spp. Because anisomycin is used in selective media for other bacteria, studies of the benefit of switching to natamycin for these media should be performed as well.

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