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Enhanced Antifungal Effect of the Selective Medium for the Detection of *Legionella* Species by a Combination of Cycloheximide, Amphotericin B and Thiabendazole

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The accurate detection of *Legionella* from environmental water samples using conventional plate culture methods is often made difficult by the overgrowth of non-target microorganisms on the selective agar plates. Acid pretreatment is a very effective pretreatment to decrease the overgrowth of heterotrophic bacteria. However, acid pretreatment would not be expected to eliminate molds. We evaluated the effects of four kinds of antifungal agents, individually and in combination, on the growth of *Legionella* strains and molds. Consequently, it was demonstrated that the combination of cycloheximide, amphotericin B and thiabendazole was very effective in eliminating molds on agar plates, and resulted in the improved detection of *Legionella*. Examination of 214 cooling tower water samples using the enhanced antifungal selective medium (CAT α) instead of GVPC α selective medium demonstrated a decrease in contamination by molds from 13.6 % to 1.9 % without affecting the growth of *Legionella*.

Key words : Antifungal agent/Detection/*Legionella*/Selective medium/Thiabendazole.

INTRODUCTION

Legionella species are gram-negative bacteria ubiquitously found in natural and man-made water systems. Human inhalation of aerosolized water from *Legionella* contaminated sources, such as cooling tower water or bath water, can result in a severe form of pneumonia called Legionnaires' disease (Vogel and Isberg, 1999). Therefore, the control and management of *Legionella* contamination in water systems is very important, and the effectiveness of these efforts must be monitored by screening for the presence of *Legionella*. There are several methods to detect *Legionella* from environmental water samples including plate culture, fluorescent antibody staining, and gene detection. Of these, the plate culture method is the standard method (ISO11731, 1998) for the detection of *Legionella*.

However, the detection of *Legionella* in environmental water samples using conventional plate culture is often inhibited by the overgrowth of non-target microorganisms on the agar plates. Therefore, pretreatment of the samples and the use of a selective medium are necessary. The samples are treated with acid buffer (Bopp et al., 1981; ISO11731, 1998), heat (Dennis et al., 1984; ISO11731, 1998) or a combination of heat and acid treatment (Kasuga et al., 1999), and the treated samples are inoculated onto agar plates containing antibiotics as selective agents. Acid treatment is a very effective pretreatment to eliminate non-target heterotrophic bacteria. We recently reported that the use of the acid-phosphate buffer instead of the HCl-KCl buffer further decreased the overgrowth of non-target heterotrophic bacteria (Inoue et al., 2004). However, an acid pretreatment would not be expected to eliminate molds because some molds are resistant to low pH, and therefore, the use of a selective medium containing antifungal agents may be effective in eliminating molds.

The addition of antifungal antibiotics such as

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amphotericin B (Greaves, 1980; Okuda et al., 1984), anisomycin (Edelstein, 1981; 1982), cycloheximide (Bopp et al., 1981; Dennis et al., 1984) and natamycin (Edelstein et al., 1996) has been reported as a method for the selection of *Legionella*. However, the detection of *Legionella* in environmental water samples is sometimes inhibited by the overgrowth of molds even when using a selective medium. From our previous results using GVPC α selective agar plates, 654 (7.8%) of 8400 water samples (collected mainly from cooling towers, whirlpool baths and spas between September 2003 and August 2004) were contaminated by molds.

In this paper, we report the effect of thiabendazole as a selective antifungal agent, and we describe an improved selective medium to eliminate the overgrowth of molds on agar plates for the detection of *Legionella* by including a combination of cycloheximide, amphotericin B and thiabendazole.

MATERIALS AND METHODS

Legionella and mold strains, and growth conditions

The *Legionella* strains used were the following: *Legionella pneumophila* Philadelphia-1 ATCC33152; *L. bozemanii* ATCC33217; *L. longbeachae* ATCC33462; *L. micdadei* ATCC33218; *L. gormanii* ATCC33297; and *L. anisa* ATCC35292. These strains were cultured on a buffered charcoal yeast extract supplemented with α -ketoglutarate (BCYE α) agar (Edelstein, 1981) at 37 °C. Ten strains of molds, which had been isolated from cooling tower water samples, were grown on a potato dextrose agar (PDA) plates (4 strains) and GVPC α selective agar plates (6 cycloheximide resistant strains. These strains which had previously inhibited our examination of *Legionella* detection), were used to evaluate the antifungal effect of each type of agar medium. The molds were precultured on PDA plates at 27 °C for 2 weeks. These mold strains were identified by microscopic observation (Malloch, 1981).

Antifungal agents

Cycloheximide (Wako Pure Chemical Industries), amphotericin B (Sigma), griseofulvin (Wako Pure Chemical Industries) and thiabendazole (Sigma) were used as antifungal agents. These antifungal agents were dissolved and diluted in dimethyl sulfoxide (DMSO) to appropriate concentrations.

Evaluation of the antimicrobial activity against *Legionella* strains

Legionella strains were precultured overnight in

buffered yeast extract broth (BYEB) (Liebers et al., 1989) at 37 °C, and 500 μ l of each broth was added to 50 ml of fresh BYEB (approximately 10^6 cells/ml). The solutions (5 μ l) of antifungal agents were mixed with the broths of *Legionella* strains (995 μ l each) in 48-well micro plates. After 3 days of incubation at 37 °C, the growth of *Legionella* was judged from the turbidity of the broth.

Selective agar plates

We prepared six types of agar plates all based on GVP α agar (BCYE α containing 3 g/liter of glycine, 5 mg/liter of vancomycin, 100000 units/liter of polymyxin B). GVPC α (GVP α containing 80 mg/liter of cycloheximide), GVPA α (GVP α containing 80 mg/liter of amphotericin B), GVPG α (GVP α containing 80 mg/liter of griseofulvin) and GVPT α (GVP α containing 40 mg/liter of thiabendazole) agar plates were tested for the ability to inhibit the growth of molds. Also, CAT α (GVP α containing 80 mg/liter of cycloheximide, 80 mg/liter of amphotericin B and 40 mg/liter of thiabendazole) agar plates were prepared as a selective medium with enhanced antifungal activity.

Evaluation of each selective medium

The spores of 10 strains of molds were suspended to appropriate concentrations in 10 ml of sterile deionized water by using a nichrome wire loop. One hundred microliters of each suspension was inoculated onto five types of agar plates (GVP α , GVPC α , GVPA α , GVPG α and GVPT α). After 8 days of incubation at 37 °C, the growth of molds on each agar medium was recorded.

Next, *L. pneumophila* cells were suspended in sterile deionized water to a concentration of approximately 10^2 CFU/ml; each mold spore suspension and *L. pneumophila* suspension were mixed. Two hundred microliters of each mixture was inoculated on four types of agar plates (GVPC α , GVPA α , GVPT α and CAT α). After 8 days of incubation at 37 °C, the growth of the molds and *L. pneumophila* on each agar medium was recorded.

Next, we compared and evaluated the growth of *Legionella* colonies on BCYE α , GVPC α and CAT α agar plates. *Legionella* strains were suspended in sterile deionized water to a concentration of approximately 10^3 CFU/ml. One hundred microliters of each cell suspension was inoculated on each of three plates (BCYE α , GVPC α and CAT α). After 8 days of incubation at 37 °C, the colonies were enumerated.

TABLE 1. Antimicrobial activity of antifungal agents against *Legionella* species.

Strain	ATCC No.	MIC value ($\mu\text{g/ml}$)			
		Cycloheximide	Amphotericin B	Griseofulvin	Thiabendazole
<i>L. pneumophila</i>	33152	>200	>200	>200	100
<i>L. bozemanii</i>	33217	>200	>200	>200	100
<i>L. longbeachae</i>	33462	>200	>200	>200	100
<i>L. micdadei</i>	33218	>200	>200	>200	200
<i>L. gormanii</i>	33297	>200	>200	>200	200
<i>L. anisa</i>	35292	>200	>200	>200	100

Enumeration of *Legionella* in environmental water samples

Legionella were enumerated according to the standard method (ISO11731, 1998). Collected water samples (214 samples of cooling tower water) were concentrated 100-fold by centrifugation. The concentrated samples were pretreated with acid buffer. In this case, 500 μl of the acid-phosphate buffer (Inoue et al., 2004) was added to 500 μl of the concentrated sample in a sterile test tube with a cap at room temperature (25°C). After 10 min, 200 μl portions were inoculated onto GVPC α and CAT α selective agar plates. The plates were incubated at 37 °C for 8 days, and *Legionella* colonies on both types of agar plates were enumerated.

RESULTS

Effect of antifungal agents on *Legionella* strains

We evaluated the effect of cycloheximide, amphotericin B, griseofulvin and thiabendazole on *Legionella* strains (Table 1). Thiabendazole showed weak activity against *Legionella* with a minimum inhibitory concentration (MIC) of 100 to 200 $\mu\text{g/ml}$, but had no effect on the growth of *Legionella* at concentrations below 100 $\mu\text{g/ml}$. In contrast, cycloheximide, amphotericin B and griseofulvin had no effect on *Legionella*.

Effect of antifungal agents against molds

Next, we tested the effect of the antifungal agents in five types of selective agar plates on the growth of molds (Table 2). Both GVP α (without antifungal agents) and GVPG α (with griseofulvin) plates were overgrown with molds. GVPC α (with cycloheximide) was able to inhibit the growth of only 2 strains of molds, since 6 of the mold strains tested were resistant to cycloheximide. In contrast, GVPA α (with amphotericin B) and GVPT α (with thiabendazole) were able to inhibit the growth of 5 and 3 strains of molds, respectively. The results in Table 2 demonstrate that amphotericin B and thiabendazole each had a different antifungal spectrum.

Effect of CAT α selective medium on the detection of *L. pneumophila*

Based on the results of the foregoing experiments, we expected to increase the antifungal effect of the selective medium by the combination of cycloheximide, amphotericin B and thiabendazole. We therefore prepared CAT α selective medium containing these antifungal agents. Table 3 shows the detection of *L. pneumophila* on 4 types of selective media. CAT α selective medium allowed the detection of *L. pneumophila* colonies without the contamination of molds. In contrast, GVPC α , GVPA α and GVPT α selective media were severely contaminated by the growth of 7, 2 and 6 strains of molds, respectively.

TABLE 2. Inhibition of mold growth on each selective medium.

No.	Strain	Growth of molds on each agar plate ^a				
		GVP α	GVPC α	GVPA α	GVPG α	GVPT α
M1	<i>Penicillium</i> sp.	++	++	—	++	++
M2	<i>Trichoderma</i> sp.	++	—	+	++	—
M3	<i>Aspergillus</i> sp.	++	—	—	++	++
M4	<i>Penicillium</i> sp.	++	++	+	++	—
M5	<i>Fusarium</i> sp.	++	++	++	++	+
M6	<i>Aspergillus</i> sp.	++	++	+	++	++
M7	<i>Aspergillus</i> sp.	++	++	—	++	++
M8	<i>Aspergillus</i> sp.	++	++	—	++	++
M9	<i>Penicillium</i> sp.	++	++	—	++	++
M10	<i>Penicillium</i> sp.	++	++	++	++	—

^a++, Overgrowth; +, growth inhibition; —, no growth

TABLE 3. Detection of *L. pneumophila* on each selective medium.

No.	Strain	Colony counts of <i>L. pneumophila</i> on each agar plate (CFU/plate)			
		GVPC α	GVPA α	GVPT α	CAT α
M1	<i>Penicillium</i> sp.	23	42	OG	52
M2	<i>Trichoderma</i> sp.	42	26	39	35
M3	<i>Aspergillus</i> sp.	19	26	OG	41
M4	<i>Penicillium</i> sp.	OG ^a	15	18	16
M5	<i>Fusarium</i> sp.	6	OG	12	44
M6	<i>Aspergillus</i> sp.	4	17	OG	39
M7	<i>Aspergillus</i> sp.	OG	22	OG	22
M8	<i>Aspergillus</i> sp.	OG	20	OG	31
M9	<i>Penicillium</i> sp.	OG	32	OG	35
M10	<i>Penicillium</i> sp.	OG	OG	35	30

^aOvergrowth of the molds

The growth inhibition of each prepared agar medium against *Fusarium* species (M5) is shown in Figure 1. Thus, the synergistic effect of a combination of cycloheximide, amphotericin B and thiabendazole against molds was demonstrated.

Next, we examined the growth of *Legionella* colonies on CAT α selective medium. Table 4 shows a comparison of the colony counts of *Legionella* on BCYE α , GVPC α , and CAT α selective media. No difference in the percentage of colony forming units

on GVPC α and CAT α selective media was observed.

Detection of *Legionella* using CAT α selective medium

From the results shown in Tables 3 and 4, the CAT α selective medium is very effective in inhibiting the growth of molds and supporting the growth of *Legionella*. Accordingly, we evaluated the validity of CAT α selective medium by comparing it with GVPC

FIG. 1. Growth inhibition of each prepared agar plate against *Fusarium* species (M5). The photographs show the growth of *Fusarium* species and *L. pneumophila* after 8 days of incubation at 37 °C. A), GVPC α , containing 80 μ g/ml of cycloheximide; B), GVPA α , containing 80 μ g/ml of amphotericin B; C), GVPT α , containing 40 μ g/ml of thiabendazole; D), CAT α , containing 80 μ g/ml of cycloheximide, 80 μ g/ml of amphotericin B, and 40 μ g/ml of thiabendazole.

TABLE 4. Comparison of the growth of *Legionella* on BCYE α , GVPC α and CAT α selective media

Strain	ATCC No.	% Recovery of <i>Legionella</i> colonies on: ^a		
		BCYE α	GVPC α	CAT α
<i>L. pneumophila</i>	33152	100	74	78
<i>L. bozemanii</i>	33217	100	10	14
<i>L. longbeachae</i>	33462	100	85	83
<i>L. micdadei</i>	33218	100	71	83
<i>L. gormanii</i>	33297	100	4	3
<i>L. anisa</i>	35292	100	40	44

^aThe value shows the percentage of colony forming units of *Legionella* (two replicate plates) when the colony counts on BCYE α were 100 %. Initial cell counts of strains in each cell suspension were the following: *L. pneumophila*, 2×10^3 CFU/ml; *L. bozemanii*, 1×10^3 CFU/ml; *L. longbeachae*, 3×10^3 CFU/ml; *L. micdadei*, 6×10^3 CFU/ml; *L. gormanii*, 4×10^3 CFU/ml; *L. anisa*, 2×10^2 CFU/ml.

TABLE 5. Comparison of mold contamination on GVPC α and CAT α selective media

Result	GVPC α	CAT α
Growth of molds	21 (9.8 %)	3 (1.4 %)
Overgrowth of molds ^a	8 (3.7 %)	1 (0.5 %)
Total	29 (13.6 %)	4 (1.9 %)

$n = 214$

^a The growth of molds over more than a third of the agar plate

FIG. 2. Comparison of *Legionella* counts detected on GVPC α and CAT α selective media ($n = 52$)

α selective medium. Table 5 shows the comparison of mold contamination on GVPC α and CAT α selective media. Out of a total of 214 cooling tower water samples, 29 samples (13.6 %) were contaminated with molds on GVPC α selective medium. In contrast, four (1.9 %) of these 214 samples were contaminated with molds on CAT α selective medium. Furthermore, of these samples, eight (3.7 %) were overgrown with molds which covered more than a third of the GVPC α agar plate. In contrast, only one sample (0.5 %) was overgrown with molds on CAT α selective medium. Figure 2 shows the number of *Legionella* colonies on the GVPC α and CAT α agar plates plotted as a scatter diagram. The samples with no colonies on both types of selective media were eliminated after this figure was plotted. From these results, a high coefficient of correlation ($R^2 = 0.84$) was seen.

DISCUSSION

The conventional plate culture method is a very important technique for the detection of *Legionella* because it is the currently used standard method. However, we need to improve the accuracy of the plate culture method.

Many colonies of molds inhibit the growth of *Legionella* on agar plates. Certain types of molds produce antibiotics that can inhibit the growth of *Legionella* on agar plates, and as a result, *Legionella* numbers may be underestimated. Therefore, antifungal agents were added to the selective agar

plates to improve the accuracy of *Legionella* detection.

Lin et al. (1999) described the addition of fluconazole to standard *Legionella*-selective medium for eliminating yeasts, and Kasuga et al. (2002) reported the addition of amphotericin B to MWY selective medium for eliminating molds. Here, we report for the first time that thiabendazole was useful as an antifungal agent in the selective medium for the detection of *Legionella*, and show the validity of combining cycloheximide, amphotericin B and thiabendazole.

In another experiment, we tested higher concentrations of cycloheximide in GVPC α agar plates for the inhibition of mold growth. However, 200 μ g/ml of cycloheximide did not inhibit the growth of molds (data not shown). Therefore, a combination of antifungal agents was tested for the inhibition of the growth of molds.

Cycloheximide and amphotericin B are known to have no activity against bacteria. This is because cycloheximide is a glutarimide antibiotic that selectively inhibits eucaryotic protein synthesis, and amphotericin B is a polyene macrolide antibiotic that binds ergosterol in the cell membrane and results in the release of potassium and other ions, disrupting the function of the cell membrane. Allen and Gottlieb (1970) reported that thiabendazole inhibits the terminal electron transport system of mitochondria. Therefore, these antifungal agents have high selective toxicity against molds.

The results in Table 4 show that the percentages of *Legionella* colony forming units on GVPC α and CAT α selective media were lower than that on BCYE α medium. These data agrees with the report of Okuda et al. (1984). Based on these results, it is assumed that the effects were not due to the antifungal agents but to the antibiotics vancomycin and polymyxin B.

We added 40 μ g/ml of thiabendazole into the medium, an addition that had no effect on the growth of *Legionella*. Higher concentrations of thiabendazole may have no effect on the growth of *Legionella*, but our results indicate that 40 μ g/ml is sufficient to inhibit the growth of molds (Tables 3 and 5).

In this study, we demonstrated that the combination of three antifungal agents with different mechanisms of action is very effective for inhibiting the growth of molds on the selective medium. We believe that use of the CAT α selective medium improves the accuracy of *Legionella* detection from environmental water samples due to the reduced contamination with molds.

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