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Safety Considerations in Handling Exoantigen Extracts from Pathogenic Fungi

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Currently, exoantigen test procedures for identifying mycelial form cultures of pathogenic molds require that the fungi being extracted be treated with thimerosal to render them safe for handling. Recent studies have demonstrated that thimerosal may not be fungicidal. In view of these reports, we investigated the effects of thimerosal and formaldehyde on a variety of exoantigen preparations. Mature mycelial form fungal cultures, including cultures of *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum* and morphologically similar fungi, were grown on Sabouraud dextrose agar slants and treated with 0.02, 0.04, and 0.08% thimerosal for 24 and 48 h and with 0.2 and 0.5% formaldehyde for 24 and 48 h. We found that 0.5% formaldehyde killed all of the fungi studied, whereas 0.2% formaldehyde permitted the growth of only one fungus; 0.02, 0.04, and 0.08% thimerosal were fungistatic. Furthermore, 0.2 and 0.5% formaldehyde and 0.08% thimerosal affected certain antigens adversely. For those investigators who prefer to use 0.02% thimerosal and to work with sterile extracts, we recommend that the procedure be modified, and we advocate sterilization of extracts by passage through membrane filters.

For many years mycologists have used thimerosal and formaldehyde as fungicidal agents in preparing various reagents (2–9). Thimerosal diluted 1:10,000 is used routinely as a preservative in sera and spinal fluids that are to be tested for antigens or antibodies. Deighton et al. (1) showed that 0.2% formaldehyde is fungicidal for the yeast forms of *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Sporothrix schenckii* after 24 h of treatment. However, thimerosal (0.01 and 0.02%) was found not to be fungicidal for the yeast forms of these fungi after 1, 2, or 3 days of treatment.

Thimerosal is used routinely to prepare exoantigens from mycelial form cultures of mycotic pathogens (4, 5, 8, 9). The present study was initiated to compare the effects of formaldehyde and thimerosal on mycelial form cultures of *B. dermatitidis*, *Coccidioides immitis*, and *H. capsulatum*.

MATERIALS AND METHODS

Mycelial form cultures were obtained from the Mycology Division, Fungus Reference Branch, Centers for Disease Control (CDC), Atlanta, Ga., and from the American Type Culture Collection.

Media and reagents. The media and reagents used were brain heart infusion (BHI) broth and agar (Difco Laboratories), Sabouraud dextrose agar (SDA; Difco); thimerosal (merthiolate, ethylmercurithiosalicylic acid sodium salt; Eli Lilly & Co. and Eastman Kodak Co.), sodium borate (Fisher Scientific Co.), and formaldehyde (37%, wt/wt; Fisher Scientific Co.).

Antisera. The antisera used were *B. dermatitidis* antiserum, lots 76-0353 (Biological Reagents Division, CDC) and X-79 (Fungus Immunology Branch, Mycology Division, CDC), *C. immitis* antiserum, lots 18311 (Serum Bank, CDC), and C4-P1 (Fungus Immunology Branch, Mycology Division, CDC), and *H. capsulatum* antiserum lots 2, 76-0317, and 77-0353 (Biological Reagents Division, CDC).

Antigens. The antigens used were *B. dermatitidis* antigen lot 77-0134 (Biological Reagents Division, CDC), *C. immitis* antigen lot 76-0113 (Biological Reagents Division, CDC) and lots Cp 1 and 2 (Fungus Immunology Branch, Mycology Division, CDC), and *H. capsulatum* antigen lot 78-0070 (Biological Reagents Division, CDC).

All cultures were grown on SDA slants for 3 to 4 weeks at 25°C. The fungi were treated with 0.02, 0.04, and 0.08% aqueous thimerosal solutions containing 1.4% sodium borate and with 0.2 and 0.5% formaldehyde solutions. Our preliminary studies showed that sodium borate had no fungicidal effect; however, this compound did act as a chelating agent that kept the thimerosal in solution. Preliminary studies also indicated that BHI agar containing 5% sheep blood (BHIB) agar was the optimum medium for the growth of fungi treated with thimerosal and that BHI medium was the optimum medium for the growth of fungi treated with formaldehyde. The thimerosal and formaldehyde solutions were allowed to remain in contact with the fungi for 24 and 48 h, after which 0.5-ml portions of the thimerosal extracts were removed and inoculated onto BHIB agar slants. The formaldehyde

TABLE 1. Fungicidal effects of 0.02, 0.04, and 0.08% thimerosal on mature fungal cultures grown on SDA agar slants after 24 and 48 h of exposure to the chemical agent

Fungi	No. of extracts with viable fungal elements on BHIB agar/no. tested						No. of mycelial mats viable on BHIB agar/no. tested					
	24-h exposure			48-h exposure			24-h exposure			48-h exposure		
	0.02% Thimerosal	0.04% Thimerosal	0.08% Thimerosal	0.02% Thimerosal	0.04% Thimerosal	0.08% Thimerosal	0.02% Thimerosal	0.04% Thimerosal	0.08% Thimerosal	0.02% Thimerosal	0.04% Thimerosal	0.08% Thimerosal
B. dermatitidis	0/8	0/8	0/8	3/8	0/8	0/8	7/8	6/8	5/8	7/8	6/8	4/8
H. capsulatum	0/10	0/10	0/10	0/10	0/10	0/10	6/10	2/10	1/10	3/10	1/10	0/10
C. immitis	4/9	1/9	2/9	5/9	1/9	0/9	9/9	9/9	9/9	9/9	9/9	9/9
Chrysosporium spp.	3/5	1/5	1/5	3/5	1/5	1/5	5/5	5/5	5/5	5/5	3/5	3/5
Arthroconidium-producing saprophytes	2/4	0/4	0/4	2/4	0/4	0/4	4/4	4/4	4/4	4/4	3/4	1/4
Positive controls	36/36	36/36	36/36	36/36	36/36	36/36	36/36	36/36	36/36	36/36	36/36	36/36

extracts were inoculated onto BHI agar slants. Then portions of each fungal mat (at least 3 mm^2) were removed and inoculated onto BHIB agar slants (thimerosal-treated cultures) and BHI agar slants (formaldehyde-treated cultures).

To determine whether formaldehyde or thimerosal affected the potency of the antigens used, we treated *B. dermatitidis*, *C. immitis*, and *H. capsulatum* reference antigens with 0.125, 0.2, 0.25, and 0.5% formaldehyde and 0.02, 0.04, and 0.08% thimerosal. As controls, these antigens were diluted with phosphate-buffered saline (pH 7.2) to give the same volumes and dilutions as the antigens diluted with formaldehyde and thimerosal. All antigens were then adjusted to the original concentration of untreated reference material with a model B-15 concentrator (Amicon Corp., Lexington, Mass.) and tested by the micro-immunodiffusion procedure.

Duplicate sets of fungal cultures on slants were treated with a 0.02% thimerosal solution for 24 h. Then 0.5-ml portions of extracts were removed from one set of cultures and inoculated onto BHIB agar. The extracts from the other set of exposed cultures were filtered through 0.45-μm membranes, and 0.5-ml portions of the filtrates were inoculated onto BHIB agar slants.

The effects of thimerosal on shake flask cultures of *B. dermatitidis*, *C. immitis*, and *H. capsulatum* grown in BHI broth on a gyratory shaker at 25°C were also determined. Heavy turbid extracts were treated with 1% thimerosal to give a final concentration of 0.02%, and the cultures were placed on the shaker for an additional 24 h. They were then removed, and 0.5-ml portions of the broth cultures were inoculated onto BHIB agar slants. All cultures transferred from extracted slants or flasks were held for at least 21 days before being discarded as showing no growth.

RESULTS

Studies were performed with 36 cultures, including 8 *B. dermatitidis* isolates, 10 *H. capsulatum* isolates, 9 *C. immitis* isolates, 5 *Chrysosporium* spp. isolates (2 *Chrysosporium parvum*, 1 *Chrysosporium keratinophilum*, and 2 *Chrysosporium* spp.), and 4 arthroconidium-producing saprophytes (1 *Arachniotus reticulatus*, 1 *Arachniotus* sp., 1 *Auxarthron* sp., and 1 *Malbranchea* sp.).

As Table 1 shows, sets of 36 slant cultures were tested with 0.02, 0.04, and 0.08% thimerosal for 24 and 48 h. Except for extracts of three *B. dermatitidis* isolates, which produced growth after 48 h of exposure to 0.02% thimerosal, none of the *B. dermatitidis* isolates demonstrated viability. Furthermore, none of the *H. capsulatum* extracts produced growth. Some of the extracts from the *C. immitis* isolates grew after exposure for 24 and 48 h to all of the concentrations of thimerosal tested, except for the extracts from cultures exposed to 0.08% thimerosal for 48 h.

Extracts of the *Chrysosporium* cultures produced growth from all the thimerosal solutions,

but more of the isolates survived in 0.02% thimerosal than in 0.04 or 0.08% thimerosal, regardless of the exposure time. Extracts of the arthroconidium-producing saprophytes produced growth only in 0.02% thimerosal after 24 and 48 h of exposure. Except for *H. capsulatum*, more than one-half of the mats of all of the treated fungi grew after exposure to any of the thimerosal concentrations. Mycelial mats of 60% of the *H. capsulatum* isolates exposed to 0.02% thimerosal for 24 h grew, whereas 30% or fewer exposed to the higher concentrations of thimerosal for 24 and 48 h grew (Table 1).

Duplicate isolates of the three pathogens and the saprophytic species were also exposed to 0.2 and 0.5% formaldehyde for 24 and 48 h. Except for one isolate of *C. parvum*, the duplicate formaldehyde-treated (0.2 and 0.5%) cultures were all killed (Table 2). The *C. parvum* isolate grew from the 48-h extract and from the mat after 24 and 48 h of exposure to 0.2% formaldehyde.

Duplicate control cultures were treated with sterile distilled water for 24 and 48 h rather than with formaldehyde or thimerosal. These controls all produced growth (Tables 1 and 2).

Since our previous experiences with exoantigen stability had been restricted to 0.02% thimerosal, it was logical to determine the effects of other concentrations of thimerosal and formaldehyde on specific diagnostic exoantigens. Reference antigens were diluted with a 1.0% solution of thimerosal to give final concentrations of 0.02, 0.04, and 0.08%. Other portions of the antigens were also treated with formaldehyde to give final concentrations of 0.125, 0.2, 0.25, and 0.5%. The 0.125% formaldehyde treatment did not affect the *B. dermatitidis* A antigen or the *C. immitis* IDHL antigen; however, 0.2, 0.25, and 0.5% formaldehyde diminished or eliminated the reactivities of both of these antigens. The antigenicity of the *C. immitis* IDHL antigen was also diminished by 0.08% thimerosal. The *C. immitis* IDCF and IDTP antigens and the *H. capsulatum* H and M antigens were not affected by any of the formaldehyde or thimerosal concentrations studied. Similarly, none of the exoantigens studied were affected adversely by 0.02 or 0.04% thimerosal (Table 3).

Because the high concentrations of formaldehyde and thimerosal used in this study affected several antigens adversely, we examined the use of filtration for obtaining sterile active exoantigens. Accordingly, extracts obtained from 20 pathogenic and nonpathogenic fungal cultures treated for 24 h with 0.02% thimerosal were cultured before and after filtration through 0.45-μm filters. Portions (0.5 ml) of these extracts were inoculated onto BHIB agar slants. Of 20 nonfiltered extracts, four (20%) contained viable

TABLE 2. Fungicidal effects of 0.2 and 0.5% formaldehyde on mature fungal cultures grown on SDA agar and exposed to formaldehyde for 24 and 48 h

Fungi	No. of extracts viable on BHI agar/no. tested				No. of mats viable on BHI agar/no. tested			
	24-h exposure		48-h exposure		24-h exposure		48-h exposure	
	0.2% Formaldehyde	0.5% Formaldehyde	0.2% Formaldehyde	0.5% Formaldehyde	0.2% Formaldehyde	0.5% Formaldehyde	0.2% Formaldehyde	0.5% Formaldehyde
B. dermatitidis	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
H. capsulatum	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
C. immiitis	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9
Chrysosporium spp.	0/5	0/5	1/5	0/5	1/5	0/5	1/5	0/5
Arthroconidium-producing saprophytes	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Positive controls	36/36	36/36	36/36	36/36	36/36	36/36	36/36	36/36

TABLE 3. Effects of thimerosal and formaldehyde on reactivities of *B. dermatitidis*, *C. immitis*, and *H. capsulatum* antigens

Organism	Specific antigen	Exoantigen activity after treatment with:[a]						
		0.125% Formaldehyde	0.2% Formaldehyde	0.25% Formaldehyde	0.5% Formaldehyde	0.02% Thimerosal	0.04% Thimerosal	0.08% Thimerosal
B. dermatitidis	A	+	±	±	±	+	+	+
C. immitis	IDCF	+	+	+	+	+	+	+
	IDHL	+	±	±	−	+	+	±
	IDTP	+	+	+	+	+	+	+
H. capsulatum	H	+	+	+	+	+	+	+
	M	+	+	+	+	+	+	+

[a] +, Well-defined precipitin band formation; ±, weak precipitin band formation; −, no precipitin band formation.

elements, whereas none of the filtered extracts produced growth (Table 4).

Studies were also performed to determine the effect of 0.02% thimerosal on broth shake flask cultures of *B. dermatitidis*, *C. immitis*, and *H. capsulatum*. All isolates of these pathogens became nonviable after exposure to 0.02% thimerosal for 24 h (Table 5).

DISCUSSION

Formaldehyde and thimerosal are used routinely as antifungal agents in the preparation of serological products (2, 6). During the last few years, exoantigen reagents prepared from mycelial form cultures and containing thimerosal have been checked routinely in our laboratory for sterility on SDA. The results of these tests have been consistently negative for evidence of viable fungal elements.

Recently however, Deighton et al. (1) reported that yeast forms of fungi treated with 0.01 or 0.02% thimerosal and cultured on blood agar contained viable cells. The results of the present study with mycelial form fungi agree with those of Deighton et al. (1) and suggest that SDA fungal cultures treated with thimerosal may remain viable.

Of the 8 *B. dermatitidis* and 10 *H. capsulatum* cultures treated with thimerosal, only the extracts of 3 *B. dermatitidis* isolates produced growth. These extracts were from cultures treated with 0.02% thimerosal for 48 h. Thimerosal was ineffective against the *C. immitis* and *Chrysosporium* spp. isolates studied. Extracts of the arthroconidium-producing saprophytes treated with 0.02% thimerosal for 24 and 48 h also produced growth. Many mycelial mat cultures remained viable after treatment with different concentrations of thimerosal (Table 1). Our data revealed that 0.02% thimerosal cannot be relied upon to sterilize fungal extracts of cultures derived from slants.

Contrary to the results with slant cultures, we found that 0.02% thimerosal was fungicidal to broth cultures. This may be explained by the fact that more effective interaction of fungal elements with thimerosal occurs in a shake culture than with a culture on an agar slant (Table 5).

Formaldehyde was very effective as a fungicide. The 0.5% formaldehyde treatment killed all of the treated cultures, and 0.2% formaldehyde was effective against all of the treated cultures except one isolate of *C. parvum* (Table 2). Unfortunately, formaldehyde inactivated two of the exoantigens studied, the *B. dermatitidis* A antigen and the *C. immitis* IDHL antigen. The possible detrimental effects of formaldehyde on antigens should always be considered when this compound is used as a fungicide. None of the exoantigens prepared from filtered thimerosal-

TABLE 4. Occurrence of viable fungal elements in filtered and nonfiltered culture extracts exposed to 0.02% thimerosal for 24 h and cultured on BHIB

Fungi	No. of isolates tested	No. of extracts with viable fungal elements	
		Nonfiltered	Filtered
Arthroconidium-producing saprophyte	1	0	0
B. dermatitidis	7	1	0
Chrysosporium spp.	4	2	0
C. immitis	4	1	0
H. capsulatum	4	0	0

TABLE 5. Effectiveness of 0.02% thimerosal as a fungicide on broth shake flask fungal cultures

Fungi	No. of isolates	No. of isolates containing viable elements after 24 h of treatment with thimerosal
B. dermatitidis	6	0
C. immitis	6	0
H. capsulatum	9	0

treated extracts of fungal cultures showed losses in antigen reactivity. Investigators who desire to eliminate the possibility of a biohazard in the exoantigen test should sterilize slant extracts by passage through membrane filters.

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