Effect of Cetylpyridinium Chloride on Pathogenic Fungi and
* Nocardia asteroides * in Sputum

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The effect of cetylpyridinium chloride (CPC) on pathogenic fungi and * Nocardia asteroides * was studied. Sputa seeded with each of 11 organisms ( * Aspergillus flavus *, * Aspergillus fumigatus *, * Blastomyces dermatitidis *, * Candida albicans *, * Coccioidiodes immitis *, * Cryptococcus neoformans *, * Geotrichum candidum *, * Histoplasma capsulatum *, * Nocardia asteroides *, * Paracoccidioides brasiliensis *, and * Sporothrix schenckii *) were treated with CPC and kept for 2, 5, and 9 days. The CPC reagent used (0.5% CPC and 0.5% sodium chloride) is one the Mycobacteriology Branch at the Center for Disease Control added to sputa before shipping them to laboratories for recovery of mycobacteria. None of the organisms tested survived this treatment, and none was recovered on mycological or mycobacteriological media. Seeded sputa containing these organisms were also tested with a second CPC reagent (0.02% CPC and 0.5% sodium chloride) and held for 2, 5, and 9 days. A few colonies of * A. flavus *, * A. fumigatus *, and * N. asteroides * were recovered from these samples. Neither the morphology of the fungi nor their stainability by the fluorescent antibody method was affected by treatment with the reagent containing 0.5% CPC. However, the background material in smears from the 0.5% CPC-treated samples retained the conjugate, and this made weakly fluorescing organisms more difficult to detect. The 0.5% CPC treatment did not alter the morphology of * N. asteroides * or its ability to be stained with Kinyoun acid-fast stain.

Pathogenic fungi are often recovered in mycobacteriology laboratories from sputum submitted for examination for mycobacteria (3, 5, 13). Frequently, these isolations provide the clue to the mycotic nature of the pulmonary disease under investigation. Consequently, the effect on pathogenic fungi of reagents and procedures used to process sputa for the isolation of the mycobacteria are of interest to medical mycologists and others concerned with the accurate diagnosis of pulmonary disease.

The Mycobacteriology Branch of the Center for Disease Control (CDC) is now investigating the addition of cetylpyridinium chloride (CPC) to sputa that are to be shipped to mycobacteriology laboratories. Findings indicate that this reagent in combination with sodium chloride (NaCl) digests and decontaminates sputa enough to laboratories but does not cause a significant loss of viable mycobacteria for up to 8 days (16). These favorable findings indicate that adding CPC reagent to sputa that are to be shipped may become a routine procedure. The primary purpose of this research was to determine whether pathogenic fungi can be recovered from CPC-treated sputa submitted for mycobacteriological examinations.

Bacterial contaminants pose a problem for mycology laboratories that deal with specimens received through the mail. If a reagent that would decrease the number of contaminants without harming the pathogenic fungi could be added to the sputum before it is shipped, the recovery of these organisms would be greatly facilitated. The findings of the Mycobacteriology Branch suggest that CPC is an effective decontaminant for sputum and that it might be used for this purpose. Consequently, another objective of this work was to determine whether the CPC reagent used to isolate mycobacteria, or possibly a lower concentration of CPC, could be used as a decontaminant in sputa to be shipped to mycological laboratories for the isolation of fungal pathogens.

No information is available on the diagnostic value of the fluorescent antibody (FA) technique for the detection and identification of

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fungi in CPC-treated sputa. Therefore, the possibility of using FA for this purpose was investigated. In addition, the value of using the acid-fast stain to detect Nocardia asteroides in treated sputa was studied.

MATERIALS AND METHODS

Fungi. Two isolates of each of 10 fungi that can cause pulmonary disease were used. These were: Aspergillus flavus, A. fumigatus, Blastomyces dermatitidis, Candida albicans, Coccioides immitis, Cryptococcus neoformans, Geotrichum candidum, Histoplasma capsulatum, Paracoccidioides brasi- liensis, and Sporothrix schenckii. Two isolates of N. asteroides were also used. All of the organisms, with the exception of one isolate of A. flavus (re- covered from a parrot with aspergillosis), had been recovered from human infections. The organisms were maintained by periodic transfer to fresh media.

Sputa. Sputa were obtained from the state health department laboratories of Georgia, Mississippi, North Carolina, and South Carolina. Personnel working in the mycobacteriology sections of these laboratories saved excess sputa from samples sent in for examination for mycobacteria. At the end of each day, excess sputa were frozen and kept at −20°C. For use in this study, the sputa were thawed, pooled in 500-ml quantities, mixed by shaking on a paint shaker for 30 min, and then centrifuged for 30 min at a relative centrifugal force of 1,130 × g to remove excess microorganisms. The pooled 500-ml quantities of sputum were then dispensed in 15-ml quantities in 50-ml centrifuge tubes and kept at −20°C until needed.

Stock concentrations. The tissue form of the dimorphic fungi was used to seed the sputum because this form is found in clinical materials. To obtain the tissue form, the dimorphic fungi, with the exception of C. immitis, were grown on brain heart infusion agar at 37°C for 7 to 14 days. Since the tissue form of C. immitis is not readily obtained in vitro, mice were used for its production. Methods for producing the tissue form of this fungus in mice are given below. Growth of the other organisms for seeding sputa was obtained by cultivating them on tubes of Sabouraud dextrose agar at room temperature for 7 to 14 days. The respective growths on the artificial media were harvested and suspended in sterile distilled water in Kolmer centrifuge tubes. In the case of N. asteroides, the organisms had to be ground in a mortar to obtain uniform suspensions. The suspensions were then centrifuged for 10 min at a relative centrifugal force of 1,130 × g. The percentages of concentrations were calculated by using the volume of packed cells per volume of suspension. The stock suspensions of each fungus and Nocardia were adjusted with sterile distilled water to a standard concentration. The standard concentration of the organisms grown on artificial media was one that, when inoculated in 0.3-ml quantities to sputum, would allow 4+ growth (30 to 40 colonies) on Sabouraud dextrose agar at room temperature when 0.2 ml of the sediment of that sputum was inoculated on media.

Animal inoculations. To obtain the tissue form of C. immitis, we inoculated each of 10 albino Swiss mice intraperitoneally with 1.0 ml of a suspension of the mycelial form of each of the two isolates of this fungus. Eight days after inoculation, the mice were necropsied, and their lungs and spleens were removed and ground in a 15-ml Ten Broeck tissue grinder. Ten milliliters of sterile 0.85% saline was added to the ground tissue infected with each isolate of C. immitis to make stock suspensions for seeding the sputum. A sufficient amount of stock suspension was added to each sputum to produce 4+ growth on Sabouraud dextrose agar at room temperature.

Reagent preparation. CPC in powder form was obtained from the Aldrich Chemical Co., Milwaukee, Wis. The sodium chloride (NaCl) was obtained from Fisher Scientific Co., Fair Lawn, N.J. The CPC reagents were prepared by dissolving CPC and NaCl in sterile distilled water at room temperature. Two CPC reagents were used in the study. The first reagent contained 0.5% CPC and 0.5% NaCl; the second contained 0.02% CPC and 0.5% NaCl. (At the time of this study, personnel in the CDC Mycobacteriology Branch had found 0.5% CPC and 0.5% NaCl to be effective for the shipment of sputum for the isolation of mycobacteria. Subsequently, they increased the concentrations to 1% CPC and 2% NaCl.)

Digestion-decontamination procedure. Ten 15-ml sputum samples were seeded with the standardized quantity of each stock fungus suspension. An equal volume of CPC reagent was added to each seeded sputum. The seeded, treated sputa were then shaken on a Vortex mixer at room temperature. At intervals of 2, 5, and 9 days, each stored specimen was shaken on a Vortex mixer, and 10 ml was removed and placed in a 50-ml centrifuge tube. From this point on, each specimen was treated according to the procedure used for the recovery of mycobacteria from specimens treated with N-acetyl-L-cysteine-sodium hydroxide (17). Accordingly, sterile distilled water was added to each sample to make the final volume 50 ml. The sample was then centrifuged, the supernatant fluid was decanted, and the sediment was resuspended in 2.0 ml of 0.2% bovine albumin and cultured on selected media. Concurrently, smears were prepared from the resuspended sediment for examination by the FA procedure.

Controls. Both positive and negative controls were used in the study. The control specimens were cultured in exactly the same manner as the test sputa. The positive controls were seeded sputa to which 15 ml of a 0.5% solution of NaCl was added in lieu of the CPC reagent. The negative control specimens were unseeded sputum to which 15 ml of a 0.5% NaCl solution was added.

Culture procedure. Each sputum sample treated with the reagent containing 0.5% CPC was cultured on selected media commonly used for the isolation of the mycobacteria and also on media used for the isolation of fungi. Sputum samples treated with the reagent containing 0.02% CPC were cultured only on the mycobacterial media. The mycobacteriological media used were Lowenstein-Jensen slants and
Middlebrook 7H-10 agar plates; the mycological media were Sabouraud dextrose agar slants, brain heart infusion agar slants, and Sabhi agar plates (the formulas for preparing each of these media are given in reference 12). Both Sabhi agar and Sabouraud dextrose agar contained chloramphenicol (0.05 mg/ml). However, the antibiotic was not added to media used to recover N. asteroides, since it is sensitive to chloramphenicol. Each sediment was cultured on 1 unit of each of these media, and 7,722 units of media were used in the study. Sputa cultured on the mycobacteriological media were incubated in 8% CO₂ (1) at 37°C in a Napco CO₂ incubator (model 322). Those cultured on Sabouraud dextrose and Sabhi agars were incubated at room temperature, and those cultured on brain heart infusion were incubated at 37°C.

Cultures were examined, and the growth was recorded each week for 4 weeks. The amount of growth was recorded as follows: 4+ (30 to 40 colonies), 3+ (20 to 30 colonies), 2+ (10 to 20 colonies), 1+ (1 to 10 colonies), and − (no growth). At the end of 4 weeks, all cultures showing no growth were considered negative.

FA studies. Ten FA reagents were used in the study. The CDC Mycology Division uses eight of these in its diagnostic service. They are: a screening conjugate for Aspergillus species (6); a screening conjugate for B. dermatitidis and H. capsulatum (11); a specific conjugate for B. dermatitidis (9); a screening conjugate for C. albicans (6); a specific conjugate for C. inmitis (7); a specific conjugate for C. immitis and H. capsulatum (10); a specific conjugate for H. capsulatum and B. dermatitidis (10); and a specific conjugate for S. schenckii (8). Two reagents used in the study, fluorescein-labeled G. candidum antiglobulins and fluorescein-labeled P. brasiliensis antiglobulins, are experimental conjugates.

Smears for FA studies were prepared from seeded sputa treated with the 0.5% CPC reagent and from the untreated seeded sputa after they had been stored for 2, 5, and 9 days. To obtain additional information on the effect of CPC on the staining of the organisms by FA, we also prepared smears from 0.5% CPC-treated saline (0.85%) suspensions and untreated saline suspensions of each organism after holding them for 2, 5, and 9 days. Two smears were prepared from each specimen. In each case, one smear was stained with the fluorescein-labeled rabbit antiglobulins corresponding to the particular organism being tested. The other smear was stained with fluorescein-labeled rabbit preimmunization globulins and served as a negative control.

The staining methods were those employed by the CDC Mycology Division; that is, the smears were allowed to dry at room temperature and then heat fixed. Later, the smears were covered with the respective conjugates and incubated in a moist chamber for 45 min at 37°C, except for smears stained with the S. schenckii conjugate, which were incubated for 30 min at 37°C. After this treatment, the smears were rinsed in phosphate-buffered saline (pH 7.2) for 10 min and in distilled water for 5 min. After the preparations had dried at room temperature, they were mounted with phosphate-buffered saline (pH 7.6) glycerol.

The fluorescence intensity of the stained organisms was rated according to the criteria of Cherry et al. (2): 4+ (maximal fluorescence; brilliant green), 3+ (bright yellow-green fluorescence), 2+ (less brilliant but definitely fluorescent), 1+ (fluorescent but dull), ± (questionable fluorescence), and − (no fluorescence).

A Reichert Biozet microscope with a Reichert Fluorex light source unit was used for examining the stained smears. The light source was an HBO 200 high-pressure, mercury-vapor lamp. The filters used in this system were a 422-1 interference exciter filter (15) and a Schott GG-9 ocular filter.

Acid-fast stain. Screening, specific, or partially specific conjugates were available for all organisms included in this study, except for N. asteroides. Reliable conjugates have not been developed for the detection or identification of the Nocardia species. Consequently, the smears prepared from CPC-treated and from untreated sputa seeded with N. asteroides after they had been held for 2, 5, and 9 days were stained with a modified Kinyoun acid-fast stain (4) to determine whether CPC affects the acid fastness or the morphology of this actinomycete.

RESULTS

None of the organisms used in this study was recovered on either the mycological or the mycobacteriological media from samples of seeded sputa treated with 0.5% CPC and held 2, 5, and 9 days. Furthermore, no organisms of any kind were recovered from the 0.5% CPC-treated sputa on the mycological media, and only mycobacteria (present in the sputa when it was received) were recovered on the mycobacteriological media.

Cultures made from sediments of samples of untreated seeded sputa held 2, 5, and 9 days produced as much as 3 to 4+ growth of each organism, with the exception of H. capsulatum. Numerous colonies (3 to 4+ growth) of both isolates of H. capsulatum were recovered from the sediments of samples held 2 and 5 days; however, neither isolate of this fungus was recovered on any media inoculated with samples held 9 days. This finding was not unexpected, because H. capsulatum has been reported to die in clinical specimens left at room temperature (4). Growth of the other fungi from sediments of samples held 9 days was not as luxuriant as that from the sediments of samples held 2 and 5 days.

All of the test organisms recovered from untreated sputa grew better on the mycological media than they did on the mycobacteriological media, except N. asteroides, which grew equally well on both types of substrates. Of the mycobacteriological media, Sabhi agar yielded the largest number of colonies. The colonies were also larger in size. Sabouraud dextrose agar ranked next for the recovery of fungi, and brain
heart infusion agar was the least efficient of the three.

Neither fungi nor *N. asteroides* was recovered from the negative control (unseeded, untreated) sputa. Only bacterial colonies were recovered.

Since the 0.5% concentration of CPC was unsuitable for recovery of fungi from sputum held at ambient temperatures, we tried to determine whether a lower concentration of this reagent might be used for this purpose. Because the 0.5% reagent killed all of the organisms, we thought that the concentration of CPC chosen should be as low as possible, yet one that would allow the reagent to function as a decontaminant. To determine this lower concentration, we treated raw sputa with varying percentages of the reagent. These experiments showed that 0.02% CPC was the lowest concentration at which the reagent would function as a decontaminant.

A few colonies of both isolates of *A. flavus* and *A. fumigatus* and numerous colonies of both isolates of *N. asteroides* were recovered from seeded sputum treated with 0.02% CPC. None of the other fungi tested survived.

In contrast to the viability of the fungi, the morphology, as determined by FA examinations, did not appear to be affected by the 0.5% CPC treatment. The intensity of the fluorescence of the stained organisms, however, appeared to be lower in smears of the treated sputum sediments than in those of the untreated sputum sediments. This difference is considered to be due to the effect of the background. Smears from CPC-treated sputum sediments had a homogenous background material that retained the conjugates and fluoresced from 1 to 2+. In contrast, smears from untreated sputum sediments the background material was not homogenous, showed minimal conjugate retention, and did not present a troublesome fluorescence. As a result, organisms in smears from untreated sputa were easier to find, and they appeared brighter than did those from CPC-treated sputa. The background fluorescence noted with CPC-treated sputa caused no practical difficulty in discerning the fungi that were stained at 3 to 4+ levels of intensity. However, the organisms that fluoresced at 1 to 2+ levels, such as *H. capsulatum* stained with the bivalent conjugate and *B. dermatitidis* stained with specific conjugate, were difficult to detect in the smears.

Further evidence that the apparent lower fluorescence of the test fungi in smears from CPC-treated sputum was due to background fluorescence was obtained with studies of seeded saline suspensions. The smears made from CPC-treated saline sediments had no background fluorescence, and the organisms in these smears fluoresced as brightly as did those in smears prepared from untreated saline sediments. In addition, the organisms in smears made from CPC-treated saline sediments fluoresced as brightly as did those in smears made from sediments of untreated seeded sputum. There was no detectable difference in fluorescence or morphology of fungi in smears made from samples held for 2, 5, and 9 days.

Neither the morphology nor the acid fastness of *N. asteroides* appeared to be affected by the CPC treatment. Organisms in smears made from CPC-treated sputum sediments stained as well as did those in smears from untreated sputum sediments. Furthermore, the background material did not retain the acid-fast stain; consequently, the organisms were easy to find in the smears. There was no observable difference in acid-fast staining or morphology of organisms in smears made from the same sputum kept at room temperature for 2, 5, and 9 days.

**DISCUSSION**

Our studies show that CPC is not a suitable reagent to add to sputum that is to be shipped or held for cultural studies for fungi or *N. asteroides*. When this reagent is used at a concentration of 0.5%, a level that has been found to give satisfactory results for the isolation of mycobacteria, the principal pulmonary mycotic disease agents are apparently killed. Therefore, if laboratory workers plan to add CPC at levels of 0.5% or higher to sputa to be shipped for mycobacteriological examination, they should also submit duplicate untreated specimens for culture for pathogenic fungi and *N. asteroides*. When CPC was used at the lower concentration of 0.02%, which approaches the limit at which CPC effectively decontaminates, a few colonies of *A. flavus*, *A. fumigatus*, and *N. asteroides* were recovered. The other fungi did not survive.

Although fungi in sputum may not survive CPC treatment, other diagnostic tests may be used to detect the presence of these agents in treated specimens. Our data show that the FA technique can be used to demonstrate the presence of fungi in CPC-treated sputum. In carrying out FA examinations on such specimens, the background fluorescence observed in stained smears is an annoyance, but it does not prevent visualization or identification of the fungi, particularly when bright-staining FA reagents are used. It may be possible to eliminate the background fluorescence. This is an
area for future investigation.

Other microscopic examinations can undoubtedly be carried out on CPC-treated sputum for the presence of pathogenic fungi and *N. asteroides*. The acid-fast stain is appropriate for *N. asteroides*. Neither the acid fastness of this organism nor the background material in the smears stained with the acid-fast stain is affected by CPC treatment.

Our study indicates that, if fungi are present in sufficient numbers, most can be recovered from untreated sputum held at ambient temperatures for up to 9 days. Even *H. capsulatum*, a relatively delicate fungus, was recovered from untreated sputum for up to 5 days in spite of increasing bacterial contamination. Unfortunately, however, sputa from patients infected with these disease agents often do not contain as large a number of fungal elements as are present in the seeded sputa used in this experiment. Consequently, it may not be possible to recover fungi from mailed sputa because of the overgrowth of contaminants. For this reason, sputa that cannot be inoculated on media before being mailed to the laboratory should either be refrigerated or treated with antibacterial antibiotics to control the growth of contaminants.

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**LITERATURE CITED**


