JOURNAL OF CLINICAL MICROBIOLOGY, May 1988, p. 827–830
0095-1137/88/050827-04$02.00/0

Vol. 26, No. 5

Congo Red as a Fluorochrome for the Rapid Detection of Fungi

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Received 17 September 1987/Accepted 28 January 1988

Congo red may be applied as a fluorochrome to rapidly detect fungi in clinical specimens, tissue, and fungal culture preparations. This generally available stain is cost effective and simple to prepare. The stain may be prepared with potassium permanganate as a counterstain or with Formalin or glutaraldehyde as a fungicide.

Various fluorescent stains with a high affinity for cellulose or chitin have been applied to stain fungal elements. Accordingly, the fluorescent brightener Calcofluor (Cellofluor, Calcofluor White M2R New, and Fungi-fluor) (6, 8, 11, 16, 17, 24; P. G. Jones, M. Hammer, and R. J. Zabransky, Clin. Microbiol. Newsl. 9:6–7, 1987) and primulin (direct yellow) (3) have been reported to stain the cell walls of plants and fungal elements in fresh or fixed histopathologic sections from human specimens. Recently, other fluorescent brighteners were demonstrated to be effective in the staining of fungi in frozen and paraffin-embedded tissues (10). Congo red was demonstrated to have an affinity for the cellulose-associated sites of various plants (8, 24), showing an intense red fluorescence.

This report discusses the use of Congo red as a nonspecific fluorochrome stain for the rapid examination and detection of fungi in fresh and paraffin-embedded tissues and of fungal isolates.

MATERIALS AND METHODS

Organisms (i) Fungi. The following fungi were obtained from our stock culture collection and were cultured on Sabouraud dextrose agar slants at 25°C: *Aspergillus fumigatus*, *Paecilomyces* sp., *Fusarium* sp., *Mucor* sp., *Rhizopus* sp., *Cladosporium* sp., *Penicillium* sp., *Phialophora verrucosa*, *Acremonium* sp., *Candida albicans*, and *Cryptococcus neoformans*. *Phialophora richardsiae* ATCC 26465 was obtained from the American Type Culture Collection, Rockville, Md. Cultures of *Exophiala jeanselmei*, *Wangiella dermatitidis*, *Fonsecaea pedrosoi*, and *Aureobasidium pullulans* were kindly provided by D. H. Pincus, Analytab Products, Plainview, N.Y. Teased preparations, slide cultures, and cellophane tape preparations were made with these organisms by standard methods (15). Congo red stain prepared with Formalin or glutaraldehyde was used to examine these fungal preparations.

(ii) Bacteria. One strain of each of the following bacteria was obtained from our stock cultures and grown on sheep blood agar, brain heart infusion agar, and chocolate agar in air at 35°C for 25 h: *Staphylococcus aureus*, *Staphylococcus epidermidis*, group A streptococcus, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter agglomerans*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Pasteurella multocida*, *Citrobacter freundii*, *Proteus mirabilis*, and *Nocardia asteroides*. The staining response with Congo red was determined for each of these organisms.

Stain reagents. (i) A 0.1% Congo red (Fisher Scientific Co., Pittsburgh, Pa.) stain was prepared in distilled water (pH 5.2). (ii) For the glutaraldehyde-Congo red stain (pH 5.0), glutaraldehyde (70%) (Polysciences, Inc., Warrington, Pa.) was diluted in distilled water to yield a 3.0% solution. Congo red was added to produce a 0.1% solution of the stain. (iii) For the Formalin-Congo red stain (pH 7.1), a 10% Formalin solution was diluted to 1% and Congo red was added to yield a 0.1% solution. (iv) For the potassium permanganate counterstain, a 0.5% solution of potassium permanganate (J. T. Baker Chemical Co., Phillipsburg, N.J.) was prepared in distilled water. One set of these stains was stored at room temperature, and a second set was maintained at 8°C.

In one experiment, 10 ml of an aqueous preparation of 0.1% Congo red in a capped test tube was placed 3 in. (ca. 8 cm) from a 100-W light source. After a 7-day exposure to the light, the stain was examined to determine its ability to react with fungal elements.

Fluorescence microscopy. The microscope used was a Leitz Ortholux with a 75-W xenon lamp and a Ploems illuminator. Specimens were examined with a 40× or 63× oil immersion lens with two combinations of Leitz filters: (i) a narrow green band excitation filter (BP530-560) and an LP 580 barrier filter that yielded an excitation maximum of 546 nm or (ii) a narrow blue band excitation filter (BP450-490) and an LP 515 barrier filter that yielded an excitation maximum of 470 nm.

Clinical specimens. Fresh clinical specimens of 40 sputum samples, 1 bile fluid sample, 11 skin scrapings, and 18 bronchial washings were each smeared on two microscope slides. After air drying, one slide was Gram stained (BBL Microbiology Systems, Cockeysville, Md.), using acetone as a decolorizer, and the other slide was stained with the Congo red stain and the potassium permanganate counterstain. These specimens were also cultured for fungi by a standard method (15).

A retrospective microscopic examination of a cerebrospinal fluid sample that contained *Cryptococococcus neoformans* was performed by mixing several drops of spinal fluid with the Formalin-Congo red stain or the glutaraldehyde stain.

Paraffin-embedded tissues. Paraffin-embedded tissues containing specific fungi were selected for a retrospective examination with Congo red stain. These tissues were sectioned, deparaffinized, hydrated, and stained with the potassium permanganate and Congo red stains. Histologic stains including hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Gomori methanamine silver (GMS) were concurrently performed with other sections of these tissues by standard procedures. The various tissues chosen contained *C. albicans*, *Aspergillus fumigatus*, *Coccidioides immitis*, or *Lecythophora mutabilis* (9, 21).

Staining procedure. The teased mounts and cellophane tape preparations were placed in a few drops of the glutaral-

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dehyde-Congo red or Formalin-Congo red stain, placed under cover slips, and then examined by fluorescence microscopy. The deparaffinized tissue sections were stained with 0.1% Congo red for 1 min, rinsed in tap water, counterstained with the potassium permanganate reagent for about 10 to 20 s, and then washed in tap water. The slides were drained and dried by light blotting. A drop of nonfluorescent immersion oil (Stephens Scientific, Denville, N.J.) was placed on the stained section, a cover slip was added, and the section was microscopically examined. In some experiments, Congo red and counterstained preparations of tissue sections were dehydrated through successively increasing concentrations of ethanol, cleared in xylene, and placed under cover slips with the histological mounting medium Permount (Fisher Scientific).

In one experiment, the H&E-, PAS-, and GMS-stained sections were restained with potassium permanganate and Congo red. For these preparations, the cover slips were removed with xylene and the sections were dehydrated through xylene and decreasing concentrations of ethanol to distilled water and stained with the Congo red reagent for 30 s.

RESULTS

Slide cultures and teased and cellophane tape preparations of all the yeasts and filamentous fungi examined in this investigation stained with either the glutaraldehyde- or Formalin-Congo red reagents yielded red fluorescence when examined with the narrow green band filter (Fig. 1) and yielded a red-orange fluorescence when the narrow blue band filter was used. The cell walls and cross-walls of the hyphal elements, as well as the hyphal tips, were especially brightly fluorescent.

A relatively dull red fluorescence was observed with the Congo red-stained *S. aureus*, *S. epidermidis*, *Streptococcus pneumoniae*, group A streptococcus, *P. aeruginosa*, and *Pasteurella multocida* strains examined in this investigation. The *E. coli*, *K. pneumoniae*, *Enterobacter agglomerans*, *Citrobacter freundii*, *Proteus mirabilis*, and *N. asteroides* strains did not yield any fluorescence with this stain.

Retrospective examination of all the deparaffinized embedded tissues containing fungi showed intense red fluorescence of the fungal elements with the potassium permanganate and Congo red stains (Fig. 2B, D, E, and F). The combination of stain and counterstain produced a barely perceptible tissue background that assisted in focusing. Collagen and elastin sites within the respective tissue sections were also barely perceptible with the potassium permanganate and Congo red preparations.

Although the various fungal elements in these tissues were also discernible with the GMS stain, they were not as evident with the H&E stain (Fig. 2A). The Congo red stain, however, was very effective for the observation of all fungal elements when applied to tissue preparations prestained with either H&E, PAS, or GMS. Furthermore, these stains could be effectively applied to tissues sections prestained with Congo red and potassium permanganate. The bright red fluorescence of fungi stained with Congo red was observed in tissue sections that were dehydrated and mounted in Permount.

From the fresh fluid specimens examined in this investigation, *C. albicans* was isolated from the 1 bile fluid and 15 sputum specimens. Gram stains revealed fungal elements in the bile and three sputum specimens. The use of Congo red and potassium permanganate reagents permitted the observation of fungal elements in all 16 specimens from which *C. albicans* had been isolated. Furthermore, fungal elements were observed in all six skin scrapings from which various dermatophytes had been isolated. Erythrocytes seen in the Gram-stained preparations of many of these clinical specimens did not fluoresce when stained with Congo red. Although the yeast cells of *Cryptococcus neoformans* were detectable with Congo red in the spinal fluid specimen, the capsules of these organisms did not appear to bind to the stain.

The fluorescent staining of fungal elements with the various Congo red preparations stored either at room temperature or 8°C appeared similar. Furthermore, these reagents remained effective after storage of 7 months. Relatively long

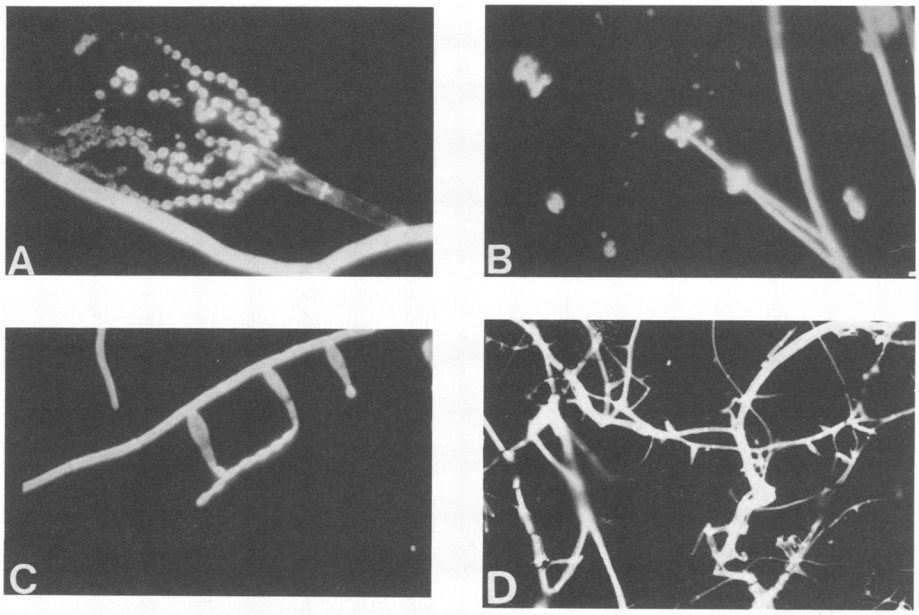

FIG. 1. Formalin-Congo red-stained slide culture preparations of *Penicillium* sp. (A), *Acremonium* sp. (B), and *Cladosporium* sp. (C) and cellophane tape preparation of *Phialophora richardsiae* (D). Magnification, ×1,000.

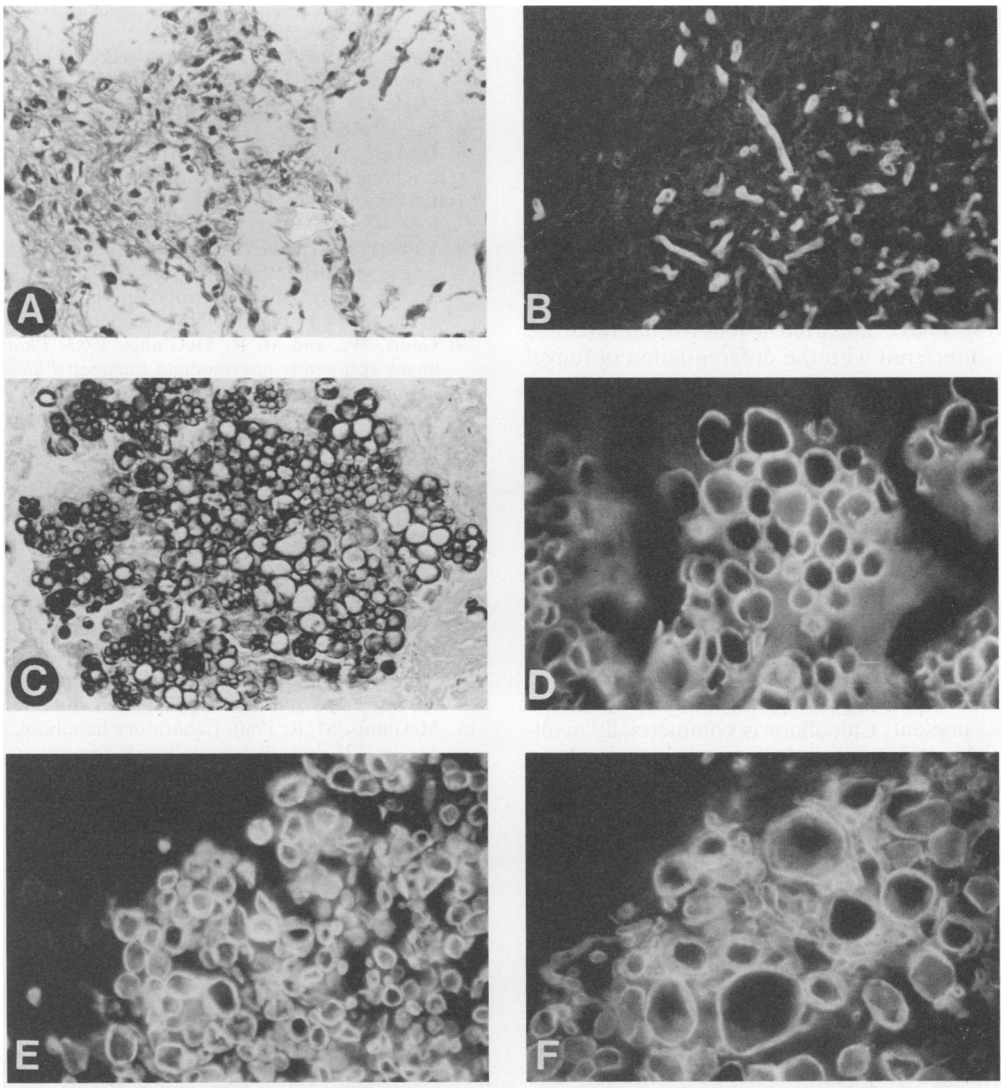

FIG. 2. Paraffin-embedded stained tissue sections containing fungi. (A) H&E-stained section of lung tissue containing *C. albicans*; magnification, ×400. (B) Potassium permanganate-Congo red-stained section of lung tissue containing *C. albicans*; magnification, ×630. (C) GMS-stained section of heart tissue containing *Coccidioides immitis*; magnification, ×400. (D) Potassium permanganate-Congo red-stained section of heart tissue containing *Coccidioides immitis*; magnification, ×630. (E) Potassium permanganate-Congo red-stained section of lung containing *Aspergillus fumigatus*; magnification, ×630. (F) Potassium permanganate-Congo red-stained heart valve vegetation containing *L. mutabilis*; magnification, ×630.

exposure of the aqueous Congo red stain to light did not alter its ability to fluorescently label fungal slide cultures or paraffin sections, compared with that of the stains maintained in the refrigerator or at room temperature on laboratory shelves. No precipitation of these stains occurred throughout the 8-month period of this investigation.

DISCUSSION

Congo red is a diazo compound that can be used as an indicator and as a specific stain for amyloid (14). Amyloid stained with Congo red yields a green fluorescence in polarized light (14). Congo red and also Calcofluor have been used to stain the endosperm cell walls of barley, oats, and wheat (8, 24). Both dyes have an equivalent reaction with beta-glucan moieties in plant structures (24). Congo red stains by both chemical linkage and physical absorption (23).

Although a recent report has disclosed that Calcofluor did not stain the spherules of *Coccidioides immitis* (20), our results show that Congo red permits the detection of these specific fungal structures in tissue sections. Calcofluor may bind to bacteria under some conditions (D. Moore and W. Schmidt, Author's Reply, Am. J. Clin. Pathol. **87**:296, 1987). The minimal fluorescence observed with some of the Congo red-stained bacteria examined in this investigation was in contrast to the bright red fluorescence associated with the fungi stained with this reagent. Accordingly, unlike the results reported for Calcofluor (1), bacteria stained with Congo red should not be misidentified as fungal elements. Furthermore, erythrocytes stained with the Congo red stain reagents did not fluoresce and therefore should not be misidentified as yeast cells (1).

Various investigations have provided data on the chemical structure of fungal cell walls (2, 4, 7, 19). The members of the dematiaceous fungi such as *Phialophora* species contain relatively small amounts of chitin in their cell walls (19). The present investigation demonstrated that Congo red effec-

tively produces bright fluorescent labeling of the cell wall structures of various *Phialophora* species and other related organisms. Although the cell wall polysaccharides of *Cryptococcus neoformans* contain chitin and glucan complexes (19), the capsular material of this organism contains mannose-associated polysaccharides (19). Accordingly, Congo red, like Calcofluor (16), did not stain these structures.

Potassium permanganate is an established counterstain in fluorescence microscopy of acid-fast bacilli (12, 22). This counterstain produced an essentially nonfluorescent background in contrast to the bright red fluorescent sites observed with Congo red. The use of Evans blue as a counterstain (11, 16) would have imparted a reddish fluorescence that would have interfered with the differentiation of fungal elements with Congo red.

This investigation demonstrated that Congo red may be used as an alternative to Calcofluor for the examination of fungi in paraffin-embedded tissues, as well as of fungal isolates and fungi in slide cultures, cellophane tape preparations, and fresh clinical specimens. Both Congo red- and Calcofluor (17)-stained preparations can be prepared in a mounting medium that consists of a synthetic resin such as Permount.

Congo red has several distinct advantages beyond those reported for Calcofluor and other fluorescent compounds (10, 24). Congo red is a cost-effective reagent, since 25 g costs about $4.00; the same amount of Calcofluor costs about $138.00. At present, Calcofluor is commercially available in diagnostic kits. Congo red, in contrast to Calcofluor, is generally available in a histological laboratory, particulary for its applications in the microscopic examination of amyloid in tissue sections (18). The Congo red reagents described in this investigation have a shelf life of at least 8 months at room temperature or in a refrigerator. The various Congo red reagents used in this investigation were effective at pH 5 to pH 7 and appear not to be light sensitive. Unlike the commercially available Calcofluor preparations, the glutaraldehyde- or Formalin-Congo red reagents afford the technologist a less biohazardous means to examine fungal preparations derived from cultures or fresh tissues. This is analogous to the fungicidal aspect of lactophenol cotton blue stain (5, 13).

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