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THE EFFECT OF AGE ON THE SENSITIVITY OF CELL CULTURES TO

CLOSTRIDIUM DIFFICILE TOXIN

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This thesis is approved as a satisfactory and independent investigation by a committee for the degree, Master of Science, and is acceptable for meeting the thesis requirement for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

BY

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A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science
Major in Microbiology
South Dakota State University
1985

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THE EFFECT OF AGE ON THE SENSITIVITY OF CELL CULTURES TO
CLOSTRIDIUM DIFFICILE TOXIN

Abstract

JO ANNE TICHOTA-LEE

Clinical microbiology laboratories performing C. difficile toxin assays usually elect to maintain only cell lines shipped from suppliers. In this study, four cell lines, primary human foreskin (HFS), Chinese Hamster Ovary (CHO-K1), HEp-2, and WI-38 were tested for sensitivity to the effects of C. difficile cytotoxin. The influence of the age of the culture and duration of the assay, 4 versus 24 hours, was also examined. Twenty-eight positive and 13 negative patient specimens were tested. Tubes of cells were inoculated at ages of 3,4,5,6,7,9, and 14 days and examined for cytopathic effects at 4 and 24 hours post inoculation.

The four cell cultures detected fewer fecal samples positive for C. difficile toxin when the assay was read at 4 hours compared to 24 hours after inoculation. However, the HFS cell line was the most sensitive in detecting C. difficile toxin compared to the other three cell lines. Five to 6-day-old primary HFS cells were 14 to 17% more sensitive than the other three cell types. Sensitivity of all four cell cultures to C. difficile toxin decreased as the age of the cell cultures increased. The HFS cells detected C. difficile toxin, in fewer

fecal samples, but were still 12-18% more sensitive at 7 days of age, at 9 days of age, 13-20% more sensitive and 12-20% more sensitive at 14 days of age than were the HEp-2, CHO-K1 and WI-38 cells. The sensitivity of all four cell lines to C. difficile toxin was greater if the cytotoxicity assay was observed at 24h after inoculation. This was especially true for fecal samples containing low levels ($\leq 1:160$ titer) of C. difficile toxin. The HFS cells were 3-7% more sensitive at 24h after inoculation compared to the other three cell cultures. However, the HFS cell line remained 100% sensitive except on days 3 and 7, when one true positive was not detected.

In conclusion, HFS, CHO-K1, HEp-2 and WI-38 cells are less sensitive to the effects of C. difficile toxin as the cultures age. Primary HFS cells are more sensitive than CHO-K1, HEp-2 and WI-38 cells for detection of C. difficile toxin in feces, regardless of the age of the cells and the time of incubation of the cytotoxicity assay.

LITERATURE REVIEW

Antimicrobial Agents Implicated

Most antimicrobial drugs and some chemotherapeutic agents have been implicated in C. difficile-induced diarrhea (3,39,57,63). Clindamycin and cephalosporins are the most frequent antibiotics involved in cases of antibiotic-associated diarrhea or pseudomembranous colitis (PMC) (3,40,57). Clindamycin-induced diarrhea or PMC occurs in 1 of 50,000 to 1 of 100,000 treatments as reported by the manufacturer (67). However, clindamycin may be involved in 10 percent of cases of antibiotic-associated diarrhea or PMC and has a mortality rate up to 44 percent in untreated patients (62). Non-toxigenic strains of C. difficile do not revert to cytotoxin production in the presence of clindamycin but 6/56 toxigenic strains produced higher levels of cytotoxin in the presence of clindamycin (43).

Antibiotic-associated diarrhea and PMC have also been documented in at least two cases of patients treated with cephalosporins (15,60). Fecal filtrates from one patient with antibiotic-associated diarrhea contained cytotoxin, which was neutralized by polyvalent gas gangrene and C. difficile antitoxins (15). A fatal case following prophylactic use of cephalosporins was reported in a 67-year-old female, who developed complications of abdominal pains and diarrhea after a hysterectomy. An autopsy of

this woman revealed lesions typical of PMC involving the ileum and the entire colon (60).

Antibiotic-associated diarrhea and PMC also develops in patients treated with other antibiotics (21,22,71). A fatal case of PMC associated with the use of neomycin and erythromycin during whole gut irrigation has been described (71). Metronidazole was used successfully in the treatment of one patient (14), but has also been found associated with PMC in another (29).

Studies to Detect an Etiologic Agent

In studies investigating a possible viral etiology of PMC, cytopathic effects (CPE) were observed in cell cultures inoculated with fecal material from patients with PMC (32). Since the CPE could not be reproduced on passage of the cell supernatant as expected with a viral agent, and the CPE was induced very rapidly, Bartlett et al.(4) concluded that the CPE was induced by a toxin rather than a virus. These same investigators had previously shown that intramuscular administration of clindamycin to hamsters caused an overgrowth of C. difficile in the cecal flora. Intracecal injections of a cell-free broth filtrate of the C. difficile strain isolated from the clindamycin-treated hamsters, induced enterocolitis in hamsters (5). In 1978, these same investigators isolated 25 clostridial strains from five fecal specimens of patients with antibiotic-associated colitis (4).

Four of these 25 strains produced CPE when inoculated onto cell cultures. Bacterial cells from pure cultures of C. difficile and broth filtrates of the four cytotoxic strains of C. difficile produced enterocolitis when inoculated intracecally into hamsters. The toxicity for cell cultures and the pathogenicity for hamsters could be neutralized by pretreatment of the filtrates with gas-gangrene antitoxin. These four cytotoxic strains were classified as C. difficile on the basis of their biological and chemical properties. Fourteen of the 21 Clostridia strains, which were not cytotoxic to cell cultures, did not produce enterocolitis in hamsters. Filtrates prepared from the stools of two patients with antibiotic-associated colitis were lethal when inoculated intraperitoneally into hamsters (50). These filtrates also produced CPE on cell cultures. The effects of the toxin on cell culture could be neutralized with C. sordellii antitoxin, but not antitoxins made against other Clostridia, Escherichia coli or Vibrio cholerae. Other investigators have reported that the cytotoxin detected in fecal samples from patients with antibiotic-associated diarrhea or PMC could be neutralized by antitoxins to C. difficile or C. sordellii (24,30,74), but not to C. histolyticum, C. novyi, C. welchii, and C. septicum (24).

C. difficile toxins are also reported to be present in fecal samples from patients with inflammatory bowel diseases (38). These toxins have been detected by a cytotoxicity assay in stools

from 17 percent of the patients with ulcerative colitis, 4 percent with Crohn's disease, and 24 percent with chronic diarrhea. Toxin was only demonstrated in those patients who had received antibiotic therapy within 2 months of sampling. Other investigators have detected C. difficile toxin in patients with inflammatory bowel disease with or without antibiotic treatment (7,9,37,66). C. difficile cytotoxin has been documented to persist in feces up to 8 months after initiation of antibiotic therapy begins (7).

Properties of the Organism and Its Toxins

C. difficile is a gram positive, spore-forming bacillus 6-8 μ m in length and approximately 0.5 μ m in width. Spores are oval, usually subterminal, and cause the bacillus to bulge minimally or not at all. Colonies of C. difficile are 2-3mm in diameter, white or grey, possess an undulant or rhizoid edge, and are slightly raised. If grown on blood agar supplemented with hemin and vitamin K, colonies of C. difficile will exhibit a chartreuse fluorescence under ultraviolet light. Colonial morphology also varies within and between strains (23).

C. difficile produces two toxins which are found in the feces of a majority of patients with antibiotic-associated colitis (2,51). One of these toxins (toxin B) produces CPE on several mammalian cell cultures (6,61). A second toxin (toxin A) causes fluid accumulation when inoculated into isolated loops of the ileum of rabbits (6).

The A and B toxins can be separated by ion-exchange chromatography on DEAE-NaCl gradients (58). Purified toxin A and partially purified toxin B are cytotoxic to Chinese hamster ovary (CHO-K1) cells (58). Antiserum to toxin B did not neutralize the ability of toxin A to produce CPE on cell cultures and vice versa. The quantity of toxin B was 1000X higher than toxin A. The specific activities of toxins A and B were 3.8×10^6 cytotoxic units (Cu) per mg and 5.3×10^9 Cu/mg, respectively. Both toxins were fatal to mice injected intraperitoneally at doses of 6.4×10^3 LD₁₀₀mg and 2.1×10^4 LD₁₀₀/mg, respectively. Toxin A and toxin B have molecular weights of 550,000 and 360,000, respectively. Both toxins are heat labile and lose 99 percent of their activity after heating at 56°C for 6 minutes. Toxin A is stable at pH 4 and 10, but labile at pH 2.0. Toxin B was labile at pH 2, 4 and 10. Both toxins are inactivated if treated with the proteases, trypsin and chymotrypsin (58).

Pathogenesis of C. difficile Antibiotic-associated Diarrhea or PMC

The pathogenesis of C. difficile-induced antibiotic associated diarrhea or PMC is not well defined. In order for C. difficile to induce diarrhea or PMC, the organism must first attach to the mucosal surface via glycoproteins, which are receptors for the bacteria on the surface of host epithelial cells. Antimicrobial drugs apparently aid the attachment of C. difficile destroying

commensal bacteria, which usually prevent the attachment and proliferation of C. difficile in the intestine (11).

Time of onset of symptoms is variable after initiating antibiotic therapy. One-half to two-thirds of the patients develop diarrhea within a few days after initiation of antibiotic treatment. Other patients do not develop diarrhea until 4-6 weeks after treatment has been discontinued (18). The clinical signs vary from a transient, self-limiting diarrhea to a severe and often fatal pseudomembranous colitis. Signs and symptoms of antibiotic-associated diarrhea include profuse watery, green feces with an offensive odor, fever, abdominal tenderness, abdominal cramps, and leukocytosis (3,18,45). Diarrhea may persist from eight to ten days, to as long as four weeks (18). Death can occur if the condition is not treated. Mortalities are highest in patients over 50 years of age. In one report, 4/21 untreated patients over the age of 50 died compared to none of 8 patients under 50 years of age (18). Recurrences are common and occur in 10 to 20 percent of treated patients (18).

Endoscopic examination of the colonic mucosa of patients with antibiotic-associated diarrhea reveals gross lesions of copious amounts of mucus covering necrotic or ulcerated areas of the colonic mucosa. Raised membranous plaques on the colonic mucosal surface are also observed and are characteristic of PMC. These plaques are yellow or green in color, vary in size and

consist of mucus, fibrin, white blood cells, and necrotic epithelial debris. As the disease progresses, the entire mucosal surface may become necrotic and slough, resulting in the formation of ulcers. Histological examinations of the colonic mucosa of PMC patients reveal a pseudomembrane consisting of a fibrinoleucocytic exudate containing polymorphonuclear leukocytes and mucin, which attaches to the surface of the colonic mucosa. The mucin is oriented as linear strands perpendicular to the surface of the colonic mucosa (3,19,45).

Epidemiology

C. difficile has been found in feces of 3 to 5 percent of "healthy" adults (19). Neonates and young infants are sometimes asymptomatic carriers of C. difficile since the cytotoxin can be demonstrated in their feces (1,59,68). Antibiotic-associated diarrhea or PMC is observed in all ages but is reported most frequently in older adult patients. It should be suspected in any patient developing diarrhea during or within six weeks after antimicrobial therapy (64). Pseudomembranous colitis was diagnosed in five children who had received prior antibiotic therapy (10). Four of these children had PMC, which clinically resembled that described in adults. Three of these children recovered and two died. C. difficile toxin has also been demonstrated in the feces of children with Hirschsprung's disease (congenital aganglionic megacolon) (65). Pseudomembranous colitis was confirmed by histopathology in two of

four specimens. However, the occurrence of Hirschsprung's disease could not be associated with antibiotic treatment and C. difficile toxin production.

Hospital environments are commonly contaminated with C. difficile (27,35,40). The extent of this environmental contamination was studied by Fekety et al.(20), who cultured various environmental surfaces in the rooms of 15 patients with antibiotic-associated diarrhea or PMC. C. difficile was isolated from the hands and feces of all patients, and from furniture used by the patients in the room. C. difficile could also be isolated from floors, toilets, bedding, mops, and scales but not samples taken from walls, windows, air conditioners, food and clean bedding. Of samples taken from various environmental surfaces, 19.6% were positive by culture for C. difficile. In comparison, only 6.8% of the samples collected from various environmental surfaces in the room of an asymptomatic carrier were positive for C. difficile and only 2.6% of the samples taken in the room of an asymptomatic, culture-negative patient were positive for C. difficile. The results of the study by Fekety (20) indicate the need for "enteric precautions"; i.e., isolation of patient and donning of gowns, gloves and masks by hospital personnel to prevent the spread of C. difficile, as suggested in the CDC Guidelines for Infection Control (CDC Guidelines for Isolation Precautions in Hospitals and CDC Guidelines for Infection Control in Hospital Personnel, U.S. Dept. of Health and Human Services, Center for Infectious Diseases, Atlanta, GA., 1983).

Nosocomial infections with C. difficile have been described (40). One patient entered the hospital with PMC, and later, four inpatients on the same ward became infected. Two of the four inpatients were given antibiotics before PMC started. In four of these five cases of PMC, C. difficile toxin was demonstrated in the feces.

Laboratory Diagnosis

Several laboratory procedures have been reported for detecting the presence of C. difficile and C. difficile toxins in feces (13,34,44,76). A cytotoxicity assay for C. difficile toxin is the preferred method because of the assay's sensitivity, specificity and good correlation with clinical diagnosis (17).

Several investigators have successfully detected the cytotoxin of C. difficile on a variety of cell lines. Ten Wen Chang et al. (13) tested 366 fecal specimens for the presence of C. difficile toxin. The test was positive in 96% of the specimens from patients with antibiotic-associated PMC and 2% from specimens of patients who were not experiencing gastrointestinal complications from antimicrobial usage. These investigators also tested the sensitivity of 7 different strains of cells to a crude toxin prepared from a strain of C. difficile and to detect C. difficile toxin in fecal extracts. The sensitivity of the different cell lines on the basis of titers of toxin in feces was determined using the Reed and Muench TCD-50 method (48). All of the cell cultures were

susceptible to the fecal extracts and the crude toxin. The most susceptible cell lines were primary human amnion, human lung fibroblast (WI-38), baby hamster kidney, and mouse fibroblasts. Three other cell lines tested (HeLa, monkey kidney and rabbit kidney cells) demonstrated titers that were approximately 10-fold lower. Murray and Weber (42) compared the sensitivity of two cell lines, Chinese hamster ovary (CHO-K1) and a human epithelial cell line (HEp-2) for the detection of C. difficile cytotoxin. They detected C. difficile cytotoxin in 26/177 (14.7%) specimens using the cytotoxicity assay. All 26 specimens were detected using CHO-K1 cells compared to 13 positive specimens detected with the HEp-2 cells. Donta and Shaffer (16) reported that C. difficile toxin produced identical cytopathic effects on Y1 adrenal cells, CHO-K1, and HeLa cells. Maniar et al. (36) found no difference in the number of specimens positive for C. difficile toxin when HeLa cells and monolayers or suspension cultures of McCoy cells were used as the assay system.

Counter immunoelectrophoresis (CIE) has also been used to detect C. difficile toxin in fecal specimens. Rennie et al. (49) compared CIE to the cell culture cytotoxicity assay and isolation of C. difficile from fecal specimens of 425 patients taking antibiotics. A total of 57 (13.4%) of the 425 fecal specimens were positive either by culture or by toxin assay. Of these, 31 (7.3%) were positive by the cytotoxicity assay, CIE and by culture, 18 (4.3%) were positive

by both the cytotoxicity assay and CIE but not by culture, 7 (1.6%) were culture positive only, and 1 was positive by CHO-K1 cell assay and negative by CIE and culture. The sensitivity of the CIE test was 80% and the cytotoxicity assay, 82% compared to the isolation of C. difficile. Standardization of the CIE test was important to eliminate nonspecific immunoprecipitation lines on the CIE assay. The CIE test was considered positive for C. difficile toxin if major precipitating lines were observed after reacting with specific C. difficile antitoxin (49). Others have encountered problems with the CIE test (31,55,72,77). West and Wilkins (72) reported finding several nonspecific immunoprecipitation arcs using CIE. They suggested that positive CIE reactions be confirmed by the cytotoxicity assay, since the C. difficile antitoxin used in the CIE procedure is prepared against a partially purified toxin, which reacts with non-toxigenic strains of C. difficile. Sands et al. (55) evaluated CIE for the detection of C. difficile toxin and found poor sensitivity in comparison to the cell culture cytotoxicity assay. They reported a sensitivity of 41%, specificity of 86% and false positive rate of 47% for CIE compared to the cytotoxicity assay. Kurzynski and co-workers (31) also reported low sensitivities in their study using two CIE procedures. The first CIE procedure used the method described by Rytel (54). The second method was similar to Rytel's with modifications (53). They found sensitivities of 33% and 47%, respectively, and specificities of 89% and 91%, respectively. Wu

and Fung (77) also reported a low sensitivity (38%) with the CIE test and a specificity of 88% as compared to the cell culture cytotoxicity assay. Prior absorption of C. difficile antitoxin with whole C. difficile bacteria reduced the number of false positive reactions on the CIE by 50% (52). If the absorbed antitoxins were used in the CIE procedure, sensitivity was 100% and specificity, 77.5% compared to the cytotoxicity test.

A selective and differential agar medium has been developed by George et al. (26) to facilitate the isolation of C. difficile from fecal specimens. This agar medium contains cycloserine, cefoxitin, fructose, and egg yolk (CCFA). They compared the ability of the CCFA and other isolation medias to support the growth of 16 strains of C. difficile. Colony counts of C. difficile grown on CCFA media were equal to the number of colonies on blood agar. Clostrisel agar, reinforced Clostridial agar plus 0.2% para-cresol, and egg yolk-neomycin agar were found to be inhibitory to the growth of C. difficile. However, when fecal specimens were cultured on the CCFA medium, it was found to be the most sensitive and selective media tested for isolation of C. difficile.

Buchanan (8) reported the use of a selective broth medium (CM&S), which contained increased levels of carbohydrates and antibiotics. Buchanan suggested that his selective broth was more sensitive than CCFA for the detection of C. difficile in fecal material, because gas chromatographic detection of volatile fatty acids from

broth cultures of C. difficile was more reliable than culture on CCFA for the detection of low numbers of C. difficile in feces.

The isolation of C. difficile from feces was enhanced in 36/60 sample cultures on taurocholate-containing medium (TCCFA) (75). The TCCFA medium substituted 0.1% sodium taurocholate for 2.5% egg yolk in the CCFA medium described by George et al. (26). The chartreuse fluorescence of C. difficile colonies was also more intense if the organism was cultured on TCCFA compared to CCFA.

Other techniques, such as gas-liquid chromatography (33), latex agglutination (56), fluorescent antibody (76), enzyme-linked immunosorbent assay (34), have also been used to detect C. difficile in fecal specimens. Gas-liquid chromatography has been used to detect the presence of volatile fatty acids and p-cresol in broth cultures of fecal material. This assay has been suggested as both unsatisfactory and satisfactory as a screening test (33,46). Shahrabadi et al. (56) found latex agglutination to have a predictive positive value of 68% and a predictive negative value of 97.5% compared to the cytotoxicity assay. A direct fluorescent antibody (FA) test agreed with bacterial culture and the cytotoxicity assay in 93% of the specimens from antibiotic-associated diarrhea patients. However, 62% of the fecal specimens from normal patients were also false positives on direct FA. The false positives may not have been a problem with antibiotic-associated diarrhea specimens because antibiotics may have eliminated the other FA-positive species of clostridia (76).

Enzyme-linked immunosorbent assay (ELISA) has been used to detect antibody to C. difficile toxins A and B (34,69,70). This assay could assess the immune status of patients with C. difficile-induced disease. It would also be a rapid and practical test for detection of toxin in feces, especially if monoclonal antibody to toxins A and B could be produced and used as capture or detection antibody in an ELISA.

Chang and Gorbach (12) developed an identification procedure combining the sensitive techniques of bacterial isolation and the cytotoxicity assay. In this assay, C. difficile was isolated on agar or in broth cultures containing cycloserine and cefoxitin. Colonies of C. difficile on the agar plates were then cut out of the agar and placed onto a cell culture monolayer to assay for toxin. A similar plug was placed onto a second monolayer containing media with C. sordellii antitoxin. A 1-ml. aliquot was also removed from broth cultures, centrifuged and inoculated onto two cell culture monolayers as described for the agar culture. The broth culture method gave more rapid and sensitive results than the agar plate culture.

INTRODUCTION

Diarrhea is a complication of antimicrobial therapy (3,11, 25). The spectrum of clinical signs associated with antibiotic-associated diarrhea varies from a self-limiting and inconsistent diarrhea to a severe pseudomembranous colitis. Staphylococcus aureus (S. aureus) was initially isolated from patients who developed antibiotic-associated diarrhea or PMC after treatment with tetracycline, chloramphenicol and oral neomycin. These antibiotics apparently suppressed the growth of normal colonic microflora, allowing an antibiotic-resistant S. aureus to become the predominant organism in the feces (11). Later, it became obvious that S. aureus was not the offending organism since in a majority of cases S. aureus is not present. However, Clostridium difficile was most frequently isolated from patients with antibiotic-associated diarrhea, and is now considered to be the principal etiological agent of these clinical diseases.

Antibiotic-associated diarrhea and PMC is mediated by the existence of two toxins, A and B, produced by C. difficile (2,51). Toxin A is responsible for the pathology induced in the colon by the bacteria but does not produce CPE on cell cultures unless present in a very high concentration (73). Toxin B produces CPE when inoculated onto several mammalian cell lines.

Inoculation of cell cultures with fecal filtrates is the best laboratory method for detection of cytotoxin produced by C.

difficile and to confirm the diagnosis of antibiotic-associated diarrhea or PMC. This cytotoxicity test relies on the ability of C. difficile toxins A and/or B to induce CPE in cultured cells.

Alternative methods such as bacterial culture, gas chromatography, enzyme-linked immunosorbent assay, and counter immunoelectrophoresis are not as sensitive as the cytotoxicity assay and do not correlate well with the clinical diagnosis. (26,33,34,55) Although the cytotoxicity assay is the preferred test to diagnose antibiotic-associated diarrhea or PMC, there are few reports on optimal standardization of this assay. Many clinical microbiology laboratories do not maintain or produce the cell cultures needed to perform the cytotoxicity assay. Such a laboratory must rely on outside sources, such as commercial companies, to provide the cell cultures needed for the demonstration of the toxin. This could mean receiving cells that are 1 to 2 weeks of age or older for use in the cytotoxicity test. Several cell lines have been evaluated and recommended for the assay of C. difficile cytotoxin (73), but data are not available on the sensitivity of individual cell cultures to the cytotoxin, the influence of the cell culture's age on sensitivity to cytotoxin, and the incubation time necessary to demonstrate CPE on the inoculated cell cultures. The purpose of the present study was to compare the sensitivity of four cell lines and to determine if the age influenced the sensitivity of the cells to C. difficile cytotoxin.

Sho-41, and WI-36 cells were obtained from a commercial source

MATERIALS AND METHODS

Fecal Specimens

Fecal samples from 41 patients with suspected antibiotic- and associated diarrhea or PMC were received at the USD School of Medicine, Clinical Virology Laboratory and frozen at -80°C until used in this study.

Fecal Filtrates

Soft feces were diluted in an equal volume of Dulbecco's phosphate-buffered saline (PBS, pH 6.9) (Flow Laboratories, Rockville, MD). Watery feces were used without prior dilution. The fecal material was then centrifuged in a Sorvall RV-3 centrifuge (DuPont Company, Wilmington, DE 19898) at $750\times g$ for 30 minutes. The supernatant was removed and filtered through a 0.45μ Type HA membrane filter (Millipore Corporation, Bedford, MA) and assayed for C. difficile toxin on cell cultures.

Cell Cultures

Four types of cells, primary human foreskin (HFS), HEp-2, Chinese hamster ovary (CHO-K1), and human fibroblasts WI-38 were evaluated in a cytotoxicity test for C. difficile toxin. Cell cultures passaged 10 times in vitro were still considered to be primary cells in this study. The HFS cells were prepared by trypsinization of human foreskins obtained from a local hospital. The HEp-2, CHO-K1, and WI-38 cells were obtained from a commercial source

(American Type Culture Collection, Rockville, MD). The CHO-K1 cell is a heteroploid cell culture originating from the ovary of a Chinese hamster (47). The WI-38 is a human, diploid, fibroblast (spindle-shaped) cell originating from the lung (28). The HEP-2 and CHO-K1 cell cultures possess squamous epithelial morphology while the HFS cells are fibroblastic. The HFS, HEP-2, and WI-38 cells were grown in Eagle's minimal essential medium (MEM) (Gibco Laboratories, Grand Island, NY) and the CHO-K1 cells were grown in Ham's F-12 medium (Flow Laboratories, McLean, VA). The media were supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY), 50mg/ml gentamicin (MA, Bioproducts, Walkersville, MD), and 4.4% sodium bicarbonate (Mallinckrodt, Inc., Paris, KY). Cells were seeded in 16x125mm glass test tubes (American Scientific Products, McGaw Park, IL) and incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cytotoxicity Assay

Each of the four cell types were evaluated at various intervals after seeding for their sensitivity to detect C. difficile toxin. One-hundred microliters of each filtrate was diluted in an equal volume of PBS. A second 100µl volume of each filtrate was diluted in 100µl of goat anti-C. difficile toxin obtained from T. Wilkins (Virginia Polytechnic Institute (VPI), Blacksburg, VA). Positive, toxin-neutralization, and negative controls were also prepared at this time. The positive control was a 1:1000 dilution of a

filtrate prepared from a culture of C. difficile which produced toxin (T. Wilkins, VPI, Blacksburg, VA). The toxin-neutralization control consisted of 100 μ l of the positive control in an equal volume of the goat anti-C. difficile toxin. Phosphate-buffered saline was the negative control suspension. All suspensions were incubated at ambient room temperature for 30 minutes to allow for neutralization of toxin in the fecal filtrates. Twenty-four hours after seeding, 100 μ l of the fecal filtrate, positive, toxin-neutralization and negative control suspensions were inoculated into an individual tube seeded with HEp-2 and CHO-K1 cells. The HFS and WI-38 cells were not inoculated at 24h because these cells have a longer population doubling time. All four cell types were inoculated at 3,4,5,6,7,9, and 14 days after seeding as described for the 24h HEp-2 and CHO-K1 cells. Cultures were incubated at 37 $^{\circ}$ C in a humidified atmosphere of 5% CO $_2$ and observed for cytopathogenic effects (rounded cells with radiating processes) at 4 and 24h post inoculation. The cell cultures were compared for their sensitivity to detect C. difficile toxin. The percent sensitivity was defined as the number of true positives (TP) divided by the number of TP plus false negatives (FN) times 100 (% sensitivity = TP/(TP+FN) X 100).

Titration of C. difficile Toxin

Fecal filtrates were diluted two or ten-fold in PBS (pH6.9). An aliquot of 100 μ l of the fecal filtrate and subsequent dilutions

were added to an equal volume of PBS; a second 100 μ l was added to an equal volume of goat anti-Clostridium difficile toxin. The mixtures were incubated at ambient room temperature for 30 minutes. Five-day-old HFS cells were inoculated with 100 μ l of the diluted fecal filtrate mixtures and the neutralized, filtrate mixtures. The HFS cells were incubated at 37°C for 48h and then examined for morphological changes due to the presence of C. difficile toxin. The titer of the toxin was expressed as the highest dilution of the fecal filtrate, which induced CPE in 50% of the cell cultures inoculated.

RESULTS

Cytopathogenic Effects of C. difficile Toxin

Twenty-seven of the 41 fecal filtrates from patients with antibiotic-associated diarrhea or PMC induced a CPE in all four of the mammalian cell lines. The absence of CPE on similar cultures inoculated with the fecal filtrate and antitoxin suspension confirmed that this CPE was due to C. difficile toxin. In addition, the CPE induced by the 27 fecal filtrates was similar to the CPE of the positive control. The cytopathic effects consist mainly of individual or clumps of rounded cells with cytoplasmic streaming (Fig. 1,2). The CPE was more readily detected in the fibroblastic cells (HFS, WI-38), which changed from a spindle shape to a rounded cell with cytoplasmic streaming (Fig. 3,4). No CPE was observed in the toxin-neutralization or negative control cultures.

The CHO-K1 cells were rounded and detached from the culture tube at 14 days after seeding (Fig. 5). This degenerative change in the CHO-K1 cells made it difficult to distinguish CPE induced by C. difficile from normal, degenerating CHO-K1 cells. The HEp-2 cells were crowded and overgrown, at 14 days after seeding, but this did not interfere with interpretation of the cytotoxicity assay. The morphology of the primary HFS and WI-38 control cells remained unchanged after 14 days in culture.

Optimum Age of Each Cell Culture at 4 and 24h Readings

When the cytotoxicity assay was read 4 hours post inoculation of the fecal filtrates, 6-day-old HEp-2 cells detected C. difficile cytotoxin in 14/28 fecal specimens (Fig. 6). Fourteen/28 specimens were positive for C. difficile cytotoxin using 7-day-old CHO-K1 cells. The 7-day-old WI-38 cells detected 16/28 positive specimens and the primary HFS cells seeded 5 and 6 days before inoculation demonstrated the cytotoxin in 22/28 fecal specimens. In general, the sensitivity of each type of cell culture decreased after reaching the peak age for sensitivity at 5 to 7 days after seeding.

In the 24-hour assay, 5 and 7-day-old HEp-2 cells detected C. difficile cytotoxin in 26/28 fecal samples (Fig. 7). The CHO-K1 cell at 4 days of cell culture age, and WI-38 at 5 days of age detected C. difficile cytotoxin in 24/28 and 27/28 samples, respectively. The HFS cells when inoculated 4,5,6,9 and 14 days from seeding detected cytotoxin in one fecal specimen, which was negative using the HEp-2, CHO-K1 and WI-38 cell lines.

- Figure 1. A. Morphology of a normal HEp-2 cell monolayer. Note the epithelial-like (polygonal shaped cells). X(500)
- B. HEp-2 cells inoculated with fecal filtrate containing C. difficile toxin. The morphological effect of the toxin on this cell line is evident by the rounding of the cells. X(500)

FIG. 1 HEp-2 Cells

A. Normal

B. Toxic

Fig. 1 HEp-2 Cells

A. Normal

B. Toxic

Figure 2. A. Morphology of normal CHO-K1 cell monolayer.

X(500)

B. CHO-K1 cells inoculated with fecal filtrate containing C. difficile toxin. Note the rounded appearance of the cells. X(500)

Fig. 2 CHO K1 Cells

A. Normal

B. Toxic

Fig.2 CHO K1 Cells

A. Normal

B. Toxic

Figure 3. A. Normal morphology of a WI-38 cell monolayer.

Note the characteristic fibroblast or spindle-shaped cells. X(500)

B. WI-38 cells inoculated with fecal filtrate containing C. difficile toxin. The morphological effect of the toxin on this cell is evident by the rounding of the cell and the cytoplasmic streaming. X(500)

Fig. 3 WI-38 Cells

A. Normal

B. Toxin

Fig.3 WI- 38 Cells

A. Normal

B. Toxic

- Figure 4. A. Normal morphology of a HFS cell monolayer. This is a spindle-shaped, fibroblast-like cell. X(500)
- B. HFS cell inoculated with a fecal filtrate containing C. difficile toxin. The cytotoxic effect is evident by the rounding of the cell and cytoplasmic streaming. X(500)

A

Fig. 4. HFS cells

A. Normal

B. Toxin

A

Fig. 4 HFS Cells

A.Normal

B.Toxic

B

Figure 5. A. Normal morphology of a CHO-K1 cell monolayer after 14 days in culture.

Note the degenerated appearance of the cells, also rounding, increased granularity, and sloughing. X(500)

B. A 14-day-old CHO-K1 cell culture inoculated with a fecal filtrate containing C. difficile toxin. Note the difficulty in distinguishing the true positive from the normal cell monolayer. X(500)

Fig. 5. CHO-K1 cell culture.

A. Normal

B. Toxin

Fig. 5 CHO-K1 Cells 14d.

A. Normal

B. Toxic

Figure 6. Frequency (number of true positives) of positive results 4 hours post inoculation.

The HFS cell detected a higher number of true positives compared to the other three cell lines. The sensitivity of each of the different cell cultures decreased after reaching maximum sensitivity to C. difficile toxin 5 to 7 days after seeding.

FREQUENCY OF POSITIVE RESULTS
4 hours post inoculation (n = 100)

Cell line age in days

FREQUENCY OF POSITIVE RESULTS
4 hours post inoculation (n = 28)

Figure 7. Frequency of positive results 24 hours post inoculation.

A higher number of positives was detected by each cell line when the assay was read at 24h. The HFS cells detected C. difficile in one fecal specimen, which was negative using the other three cell lines.

FREQUENCY OF POSITIVE RESULTS

24 hours post inoculation (n = 28)

Cell line age in days

FREQUENCY OF POSITIVE RESULTS
24 hours post inoculation (n = 28)

The Effects of Age on the Sensitivity of Cell Cultures for
Detection of C. difficile Cytotoxin in a 4h vs 24h Cytotoxicity
Assay

The four cell cultures were less sensitive when the assay was read at 4 hours compared to 24 hours after inoculation. However, the predictive value of a positive test was 100% even at 4 hours. The HFS cells were more sensitive in the 4-hour assay than the other three cell lines (Table 1). Generally, the sensitivity of the cell cultures to C. difficile cytotoxin decreased as the age of the cell cultures increased. When the cytotoxicity assay was read 4 hours post inoculation, the HFS cell detected the largest number of fecal samples positive for C. difficile regardless of age compared to the other three cell lines. Five to 6-day-old primary HFS cells were the most sensitive for detection of C. difficile cytotoxin. At this age, the HFS cells were 14 to 17% more sensitive than the other three cell types. Beyond 6 days of age, the HFS cells detected fewer fecal samples with C. difficile cytotoxin but were still 12-18% more sensitive at 7 days of age; 13-20% more sensitive at 9 days of age and 12-20% more sensitive at 14 days compared to the HEp-2, CHO-K1 and WI-38 cells.

Reading the cytotoxicity assay at 24 rather than 4 hours after inoculation of the cell cultures with fecal filtrates increased the percent sensitivity of the four cell lines (Table 2).

TABLE 1. Sensitivity^a of Cell Cultures 4 Hrs. Post Inoculation

Time from seeding of cell cultures (days)	<u>Cell Cultures</u>			
	HEp-2	CHO-K1	WI-38	HFS
1	62%	62%	ND ^b	ND ^b
3	62%	61%	67%	80%
4	64%	65%	67%	78%
5	65%	65%	68%	82%
6	67%	65%	68%	82%
7	62%	67%	68%	80%
9	58%	59.5%	65%	78%
14	54%	59.5%	62%	74%

^aSensitivity defined as the number of true positives divided by the number of true positives plus the false negatives.

^bNot done.

TABLE 2. Sensitivity of Cell Cultures 24 Hrs. Post Inoculation

Time from seeding of cell cultures (days)	<u>Cell Cultures</u>			
	HEp-2	CHO-K1	WI-38	HFS
1	87.5%	93%	ND ^a	ND ^a
3	90%	82%	93%	97%
4	90%	87%	93%	100%
5	93%	85%	97%	100%
6	90%	85%	93%	100%
7	93%	85%	87%	97%
9	90%	82%	82%	100%
14	80%	82%	85%	100%

^aNot done

The HFS cell culture was still 3-7% more sensitive than the other three cell cultures studied. As observed with the 4h cytotoxicity assay, the older cell cultures were less susceptible to the effects of the C. difficile cytotoxin. However, the primary HFS cell line remained 100 percent sensitive except on days 3 and 7 when one true positive was not detected (Table 2). The HEp-2 cells were most sensitive to C. difficile cytotoxin between 3 and 9 days of age. The CHO-K1 and WI-38 cells were most sensitive at 1 and 5 days of age, respectively.

Titration of C. difficile Cytotoxin

The titers of the cytotoxin in the fecal filtrates from the 28 positive samples ranged from 1:2 and 1:2560. The results are shown in Table 3. The primary HFS cell was more sensitive than the other three cell cultures for the detection of C. difficile cytotoxin with a titer $\leq 1:160$, regardless of the age of the cell or the time of observation (Fig. 8,9). However, the older the cells, the less sensitive was the 4h assay (Fig. 8).

When the C. difficile cytotoxin titer was $\geq 1:320$, the HFS cell was still more sensitive than HEp-2, CHO-K1, and WI-38 cells when the cytotoxin assay was read 4h post inoculation (Fig. 10). Sensitivities of all 4 cell types were similar when read 24h post inoculation except that 5-day-old CHO-K1 cells failed to detect cytotoxin in 1/28 samples. Nine-day-old HEp-2 cells also did not detect one toxin positive fecal sample (Fig. 11).

TABLE 3. Titers^a of C. difficile Cytotoxin in Fecal Filtrates

Titer of Cytotoxin	Number of Fecal Filtrates
1:2	5
1:8	2
1:10	2
1:40	1
1:80	2
1:160	7
1:320	3
1:640	3
1:1800	1
1:1280	1
1:2560	1

^a Titer of toxin was expressed as the highest dilution of the fecal filtrate which induced cytopathic effect, in 50% of all the cultures inoculated.

Figure 8. Sensitivity of cell cultures 4 hours post inoculation with fecal filtrates containing C. difficile cytotoxin titer $\leq 1:160$.

The HFS cell was more sensitive than the other three cell lines regardless of age of the cell.

SENSITIVITY OF CELL CULTURES
4 hours post inoculation, titer = 1: 160

Figure 9. Sensitivity of cell cultures 24 hours post inoculation with fecal filtrates containing C. difficile cytotoxin titer $\leq 1:160$.

Reading the assay at 24h increased the sensitivity; however, age did not affect the sensitivity of the HFS.

SENSITIVITY OF CELL CULTURES
24 hour post inoculation, titer = 1:160

Figure 10. Sensitivity of cell cultures 4 hours post inoculation with fecal filtrates containing C. difficile cytotoxin with a titer $\geq 1:320$. The HFS cell was more sensitive in comparison to the other three cell lines.

SENSITIVITY OF CELL CULTURES
4 hours post inoculation, titer 1:320

Figure 11. Sensitivity of cell cultures 24 hours post inoculation with fecal filtrates containing C. difficile cytotoxin with titers of $\geq 1:320$.

SENSITIVITY OF CELL CULTURES
24 hours post inoculation, titer = 1:320

Cell line at 5, 9, and 14 days of age

DISCUSSION

Human lung fibroblast, CHO-K1, HEp-2, HeLa, McCoy, primary human amnion, monkey and rabbit kidney and Y1 adrenal cells have been used in cytotoxicity assays for C. difficile toxins (13,16,36, 42). However, few reports have determined if the age of the cell culture influences the sensitivity of the cytotoxicity assay. Most clinical microbiology laboratories lack the time, expertise or materials available to prepare primary cell cultures. Thus, most of these laboratories commercially purchase cell cultures needed to perform the cytotoxicity assay for C. difficile toxin. This could mean using cell cultures, which are one to two weeks old, since time must be allowed for shipping and acclimatization of the cells to the environment of the recipient laboratory. The present study established that 1) different types of cells vary in their sensitivity to C. difficile cytotoxin; 2) the sensitivity of each cell culture decreased as the age of the cell culture increased and 3) the overnight (24h) cytotoxicity assay was more sensitive than a rapid 4h assay for detection of C. difficile cytotoxin in fecal filtrates.

The four cell lines, HFS, HEp-2, CHO-K1, and WI-38 cells proved sensitive to the effects of C. difficile cytotoxin. The CPE observed in each cell type was confirmed to be caused by the C. difficile cytotoxin since the effect could be neutralized by

use of goat anti-C. difficile toxin. The changes in cell morphology induced by the cytotoxin in this study were also similar to those described previously for other cell types (13). Primary HFS cells were the most sensitive to the effects of the C. difficile cytotoxin. The sensitivity of this cell line was not influenced by the age of the culture or the time of the assay in comparison to the other three cell lines.

The enhanced sensitivity of the human foreskin cells may be related to the low number (≤ 10) of subcultivations. Therefore, these cells more closely resemble cells in vivo. The HFS cells are also fibroblastic cells (elongated, spindle-like) and the rounding CPE induced by C. difficile cytotoxin on fibroblast cells is more readily observed. This easily detectable rounding of fibroblastic cells was also noticed on WI-38 cells. These morphological changes (rounding) are more difficult to notice on the epithelial cell types, CHO-K1, and HEp-2.

The increased sensitivity of the primary HFS cells compared to the stable cell lines is similar to a previous report (13) in which primary human amnion cells were found to be more sensitive to C. difficile cytotoxin than HeLa, monkey kidney or rabbit kidney cells. Thus, primary cells are more sensitive to the effects of the C. difficile cytotoxin.

Murray and Weber (42) reported the CHO-K1 cell to be significantly more sensitive than the HEp-2 cell. Our study found only

modest differences between these two cell lines. Whether the increased sensitivity observed by Murray and Weber (42) was due to use of a cell culture of minimal age or to other factors could not be determined from the published report.

Chang et al. (13) compared the sensitivity of several cell lines and primary human amnion cells for the detection of C. difficile cytotoxin. The primary human amnion cells were tested after cultivation for 1,2,3 and 4 weeks, but no mention was made as to the age of the other cell lines. His study showed that primary human amnion cells were more susceptible than HeLa, monkey kidney, or rabbit kidney cells for the detection of cytotoxin in fecal material. However, the study showed only a modest variation in sensitivity compared to other established lines. Our study showed that the age of the cell culture was very important in achieving the highest possible sensitivity. We found that cells in culture beyond 5 days were not as sensitive as cells inoculated at earlier times. The available reports in the literature make comparisons of the sensitivity of different types of cells to C. difficile cytotoxin difficult to interpret, since the age of the cell culture used is not reported.

When the cytotoxicity assay was read 4 hours post inoculation, the sensitivity of each cell culture decreased as the cell culture age increased, regardless of the cytotoxin titer. This was also true when the assay was read at 24 hours except the WI-38 and

HFS cells remaining 100% sensitive from 3 to 4 days of age. The reason for the loss of sensitivity is not known, but could perhaps relate to membrane changes that may occur with cell senescence. For example, CHO-K1 cell cultures round up and slough as they age. This makes it difficult to determine if the cytopathic effects were due to C. difficile cytotoxin or simply to degeneration of the normal cell monolayer. Failure to use negative cell culture controls on cell lines such as the CHO-K1, which degenerate with age, could result in false positive readings.

Many investigators do not report the time the assay is read. Chang et al. (13) reported that cells inoculated with a high titered toxin will show cytopathic changes within 4 hours. In our study, the primary HFS cell inoculated 5 days after seeding detected C. difficile toxin in 76% of the fecal filtrates if the cytotoxin titer was 1:160 or less. When titers were 1:320 or greater, the HFS cells were 100% sensitive. This indicates that the rapid 4h assay is best suited for fecal samples with high concentrations of the C. difficile cytotoxin. We were able to increase the number of positive readings using a 24h assay, which was more sensitive and allowed time for the lower levels of cytotoxin to produce CPE. Thus, induction of CPE by fecal filtrates containing low titers ($\leq 1:160$) of C. difficile toxin is time dependent.

The preferred test for laboratory confirmation of the cytotoxin produced by C. difficile is the demonstration of the toxic

effect of fecal extracts on cell cultures (13,16,36,42). Results from the present study indicate that primary cells, specifically, HFS cells 5 to 7 days after seeding, were most sensitive. The cytotoxicity assay should be read at 24h after inoculation. Other primary cell types may also be sensitive to C. difficile cytotoxin, such as the primary human amnion cells reported by Chang et al. (13). We did not compare sensitivities of various primary cell types so it is not possible at this time to determine if one primary cell type is more sensitive than another.

Recommendations from this study, which other clinical laboratories may consider to optimize the cytotoxicity assay for C. difficile toxin are:

1. If tissue culture facilities are available, primary HFS cells 5 to 7 days after seeding should be used. If tissue culture facilities are not available, it would be advantageous to find a commercial company or another clinical laboratory which could provide primary HFS cells and then use them up to two weeks of cell culture age.

2. Primary human foreskins can be easily prepared if the clinical laboratory has access to a normal newborn nursery. Using the foreskin cell would be a definite advantage compared to the methods necessary to obtain specimens for the preparation of primary human amnion cells.

3. If it is not possible to prepare HFS cells, an alternative choice would be to use WI-38 cells. The supplier should provide information as to the day the cell culture was seeded.

4. The assay should be inoculated in the morning hours and read 4 hours post inoculation. Although the 24-hour assay is more sensitive, 5-day-old HFS cells inoculated with fecal specimens of low titer detected toxin 76% of the time at 4 hours. In a clinical setting, even 76% is important in providing a rapid diagnosis to the clinician. Fecal filtrates negative for C. difficile cytotoxin after 4h should be incubated and observed again at 24 hours after inoculation.

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