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Diagnosis of Cryptococcosis: Comparison of Various Methods to Detect Cryptococcus Neoformans

Diagnose der Cryptpococcose: Vergleich verschiedener Methoden zum Nachweis von Cryptococcus neoformans

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Summary: The diagnosis of cryptococcal meningitis may be difficult to establish when few organisms are present in the cerebrospinal fluid. For this reason, we determined which of several different visual and cultural methods were the most sensitive for the detection of Cryptococcus neoformans. Gram stain and India ink preparations required that 10^5 organisms per ml be present before these methods were reliably positive; centrifugation increased the sensitivity of these assays 10-fold. Culturing a loopful of the sample required that 10^3 organisms per ml be present to yield growth; centrifugation of the sample enhanced the sensitivity of the culture by 100-fold. Clearly the most sensitive assays, both detecting fewer than 1 organism per ml, were culture of a membrane filter through which the entire sample had been passed and culture of the entire sample in a flask using biphasic medium.

Zusammenfassung: Die Diagnose der Crytococcus-meningitis kann Schwierigkeiten bereiten, wenn nur wenige Erreger im Liquor cerebrospinalis vorhanden sind. Aus diesem Grunde wurden Untersuchungen durchgeführt um festzustellen, welche optische oder kulturelle Methode die höchste Nachweisempfindlichkeit für Cryptococcus neoformas besitzt. Die Gramfärbung und die Aufschwemmung in chinesischer Tube benötigen 10^5 Zellen/ml um verläßlich positive Befunde zu ergeben. Durch Zentrifugierung des Liquors konnte die Empfindlichkeit auf das 10-fache gesteigert werden. Bei der Abimpfung einer vollen Impföse war es erforderlich, daß das Untersuchungsmaterial mindestens 10^3 Organismen/ml enthielt, damit in der Kultur Wachstum entstand.

Mit der Zentrifugierung des Materials wurde die Empfindlichkeit bei dieser Methode auf das 100-fache erhöht. Als empfindlichste Nachweismethoden erwiesen sich die Membranfiltermethode, bei der die gesamte Probe filtriert und anschließend kultiviert wird, sowie die Kultivierung der gesamten Probe in einer Kulturflasche mit biphasischem Nährmedium. Mit beiden Methoden lassen sich weniger als ein Organismus/ml entdecken.

Introduction

The diagnosis of cryptococcal meningitis is established definitively when Cryptococcus neoformans is isolated from the cerebrospinal fluid (CSF). However, there are patients with a cli-

nical picture of cryptococcosis in whom cultures remain negative and in whom the diagnosis is difficult to confirm. In view of the fact that organisms may be present in CSF in concentrations as low as 1 organism per 15 ml (11), some authors recommend culturing a large volume of CSF to increase the recovery rate of the organism (5). The manner in which this increased volume of CSF should be cultured has not been studied systematically. In order to determine the most efficient means of detecting C. neoformans, we compared the sensitivity of five different culture methods, the India ink preparation, and the gram stain.

Materials and Methods

Organisms. Two different strains of. C. neoformans originally isolated from two different patients with meningitis were used for these studies. They were grown on chocolate agar with hemoglobin and 1% isovitalex (BBL, Inc., Cockeysville, MD) in a 5% CO_2 atmosphere to encourage capsule formation (9). Both strains formed generous capsules when subcultured every 2 days on this medium. For the studies detailed below, the organisms were grown overnight in yeast nitrogen base (YNB) broth (Difco, Inc., Detroit, MI) supplemented with L-asparagine and glucose (10). Serial 10-fold dilutions in 0.9% NaCl were performed yielding concentrations ranging from approximately 1×10^8 organisms/ml to less than 1 organism/ml. Pour plates using Sabouraud's dextrose agar and colony counts at each dilution were performed to determine the exact concentration of organisms, expressed as colony forming units (CFU) per ml.

Gram stain and India ink preparations: Gram stain and India ink tests were made on the following: a drop from each dilution of organisms, from the sediment of each dilution centrifuged at 250 x g for 15 minutes and from the sediment of each dilution centrifuged at 1000 x g for 60 minutes. These assays were performed 15 times for each dilution of the two test organisms.

Five different culture methods were used: 1) Following thorough mixing, each 10-fold dilution was plated directly on Sabouraud's dextrose agar (Difco, Inc.) using 2 loopfuls, delivering a total of 0.02 ml. 2) 5 ml of each dilution was centrifuged at 250 x g for 15 minutes. The supernatant was discarded, and the entire sediment (about 0.5 ml) was aspirated with a Pasteur pipette and inoculated onto Sabouraud's dextrose agar. 3) 5 ml of each dilution was centrifuged at 1000 x g for 60 minutes, and the entire sediment was aspirated with a Pasteur pipette and inoculated onto Sabouraud's dextrose agar. 4) 5 ml of each 10-fold dilution was passed through a 0.45 µm millipore filter (Millipore, Inc. Bedford, MA). The filter was then removed and placed uppermost side down on a Sabouraud's dextrose agar plate. 5) The final culture method involved inoculation of the entire 5 ml sample into 2 types of biphasic media, one containing 5 ml of Sabouraud's broth with 12 ml of Sabouraud's agar and the other 5 ml of brain heart intension broth (BHI) (Difco, Inc.) with 12 ml of BHI agar. Fifty ml plastic flasks with screw caps (Corning, Inc., Corning, NY) were used for the biphasic media. After incubation of all cultures at 37°C for 24 hours, the presence of growth was determined at each dilution for each of the different culture methods. All culture assays were performed on 20 different occasions.

Results

Visual methods proved to be the least sensitive means of detecting C. neoformans. For an unspun specimen, 100,000 organisms/ml were necessary for the observer to detect 1 organism every one or two high power fields (HPF) using either India ink or Gram's stain (mean number detected was $0.7 + 0.3$/HPF) (Table 1). Centrifugation increased the sensitivity of the visual methods ten-fold so that 1 organism/HPF could be detected in a specimen with an original concentration of 10,000 organisms/ml using either of the two time-speed relation-

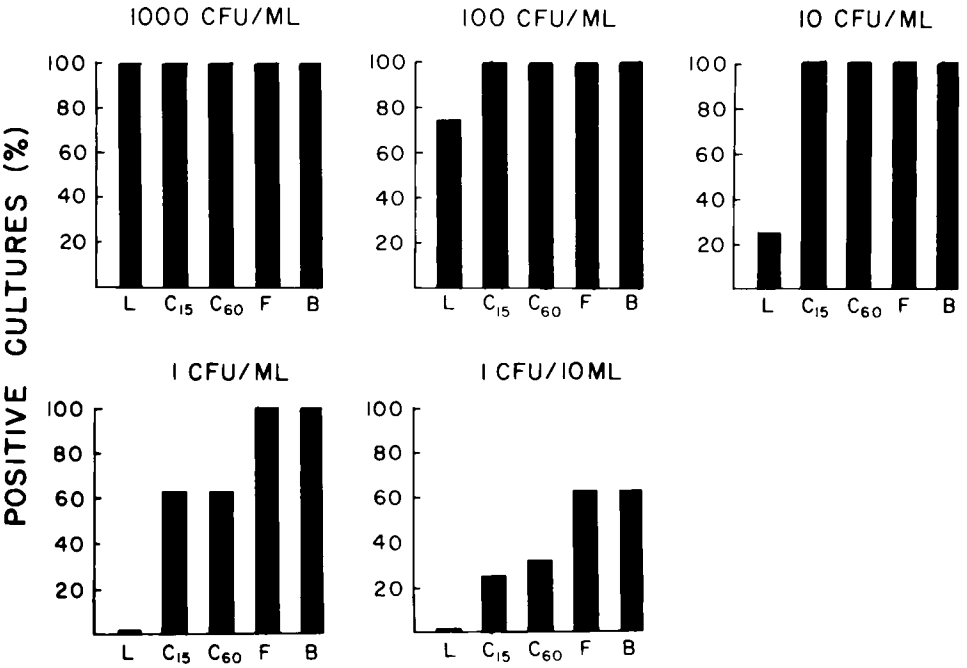

Fig. 1: Sensitivity of various culture methods for the detection of Cryptococcus neoformans. Headings denote final concentration of organisms (CFU/ml) at various dilutions studied. Culture methods include: 2 loops streaked on agar plate (L); culture of sediment after centrifugation at 250 x g 15 min. (C_{15}); culture of sediment after centrifugation at 1000 x g 60 min. (C_{60}); filtration of entire sample through millipore filter (F); and culture of entire specimen in biphasic medium (B).

Table 1
Summary of sensitivity of various methods to detect Cryptococcus neoformans

Method	Minimum number of organisms/ml required for detection
Gram stain/India ink	100,000
Gram stain/India ink following centrifugation	10,000
Culture methods	
Loopful	1,000
Centrifugation, 250 x g, 15′	10
Centrifugation, 1000 x g, 60′	10
Millipore filter	<1
Biphasic medium	<1

ships. The India ink preparation was much more easily read than the Gram's stain preparation. With the latter, staining was erratic, and the organisms were difficult to identify. With either test, if the observer spent 20–30 minutes surveying several slides, organisms could be detected occasionally at a concentration of 1000 organisms/ml.

The five different culture techniques varied widely in their sensitivity (Figure 1 and Table 1). Culturing a 0.02 ml specimen from 5 ml of broth was very insensitive, requiring 1000 organisms/ml to yield positive results on all occasions. At 100 CFU/ml, this method showed only

75% sensitivity. Centrifugation at 250 x g for 15 minutes or at 1000 x g for 60 minutes enhanced the yield 100-fold, detecting as few as 10 organisms/ml in all assays. However, centrifuged specimens were positive only 60% of the time at 1 CFU/ml. Clearly, the most sensitive methods were culture of the millipore filter, through which the whole specimen had been passed, or culture of the entire specimen in a flask with biphasic medium, either BHI or Sabouraud's media. Both methods detected fewer than 1 organism/ml and appeared to be limited only by the design of the experiment, in which 5 ml aliquots were cultured. BHI and Sabouraud's media showed no differences in sensitivity for detecting C. neoformans.

Discussion

These studies were undertaken in an attempt to find the most sensitive assay for the diagnosis of cryptococcal meningitis. The Gram's stain and the India ink preparations were relatively insensitive. In addition, the Gram's stain technique stained the organisms poorly, rendering the slides very difficult to read. This poor staining has been noted previously and is very likely due to the organism's large capsule (2). Previous studies have noted that the level of detection of bacteria by visual means appears to be approximately 1×10^5 organisms/ml, similar to our data with C. neoformans (7). The insensitivity of the India ink preparation can be inferred from several large clinical studies of cryptococcal meningitis in which this test was positive in from 57% to 64% of cases of culture-proved infection (3, 4).

The sensitivity of detecting C. neoformans by India ink and Gram stain preparations was increased by centrifugation, parelleling the observation that the yield of detecting bacteria in CSF is enhanced by centrifugation (8). There was no difference noted between centrifugation at 250 x g for 15 minutes or at 1000 x g for 60 minutes. Thus, visual methods may be adeqaute after centrifugation if the concentration of organisms in the body fluid is $\geqq 1 \times 10^4$ CFU/ml.

The culture methods were clearly superior to visual methods; however, if a direct preparation is positive, the method of culture is relatively unimportant. However, when dealing with a low concentration of organisms in the CSF, it is very important to use other than routine culture methods. Of the methods studied, the filter method and the biphasic media were the most sensitive, and the sensitivity of these two assays was limited only by the volume of fluid cultured. Culturing of larger fluid volumes has lead to recovery of the organism in patients with repeatedly negative cultures (1, 6). When dealing with cases which are negative by routine methods, the clinical laboratory must know the most efficient means of culturing a larger volume of CSF. Our results suggest that either the millipore filter method or culturing of the entire specimen in a flask with agar/broth media is the most sensitive method of recovering a small number of organisms from a large specimen volume.

References

1. Berger, M. P. & J. Paz (1976): Diagnosis of cryptococcal meningitis. J. Am. med. Ass. 236, 2517-2518.
2. Bottone, E. J. (1980): Pitfalls in diagnosis through evaluation of gram stained smears of purulent exudates. J. Clin. Microbiol. 12, 790-791.
3. Butler W. T., D. W. Alling, A. Spickard & J. P. Utz (1964): Diagnosis and prognostic value of clinical and laboratory findings in cryptococcal meningitis. N. Engl. J. Med. 270, 59-66
4. Diamond, R. D. & J. E. Bennett (1974): Prognostic factors in cryptococcal meningitis. Ann. Intern. Med. 50, 176-181.
5. Ellner, J. J. & J. E. Bennett (1976): Chronic meningitis. Medicine 55, 341-369.
6. Gonyea, E. F. (1973): Cisternal puncture and cryptococcal meningitis. Arch. Neurol. 28, 200-201.
7. Kunin, C. M. (1961): The quantitative significance of bacteria visualized in the unstained urinary sediment. N. Engl. J. Med. 265, 589-590.

8. Murray, P. R. & C. M. Hampton (1980): Recovery of pathogenic bacteria from cerebrospinal fluid. J. Clin. Microbiol. 12, 554–557.
9. Rippon, J. W. (1974): Cryptococcosis and other yeast infections. In: Medical Mycology. The Pathogenic Fungi and the Pathogenic Actinomycetes. W. B. Saunders, Philadelphia, PA, p 221.
10. Shadomy, S. (1969): In vitro studies with 5-fluorocytosine. Appl. Microbiol. 17, 871–877.
11. Utz, J. P. (1966): Fungal infection of the central nervous system. Clin. Neurosurg. 14, 86–150.

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Buchbesprechung

H. Remmert: **Ökologie** – Ein Lehrbuch. X, 304 Seiten mit 189 Abb. und 12 Tab. Springer Verlag – Berlin – Heidelberg – New York, 1980. Geheftet DM 44,–.

Seitdem E. Haeckel im Jahre 1866 den Begriff „Ökologie" prägte, war eine neue biologische Disziplin ins Leben gerufen, die sich in den nachfolgenden Jahren nur mit Mühe neben den klassischen Teilgebieten behaupten konnte. Die Gründe liegen auf der Hand und werden treffend durch die Worte Remmerts beschrieben: „Sie (die Ökologie) ist eine strenge Naturwissenschaft, sie hat es jedoch wesentlich schwerer als Physiologie, Genetik oder Biochemie: Sie muß mit einer Fülle verschiedener Parameter arbeiten, und damit werden Voraussagen unendlich schwer... Der Ökologe steht vor dem Problem, die Reaktionen und die Entwicklungen komplexer Systeme, in denen außerordentlich viele genetisch verschiedene Mikroorganismen, Pflanzen und Tiere leben, vorausbestimmen zu sollen. Der Versuch allein scheint unmöglich." Dennoch stellte der Mensch sich diesen Problemen, nicht zuletzt, da er seine eigene Existenz als biosoziales Wesen in Gefahr sah. Der Rückgang vieler Tier- und Pflanzenarten, die Vernichtung oder Bedrohung vieler Ökosysteme, der Grad der Luft- und Wasserverschmutzung, um nur einiges zu nennen, machten multifaktorielle Betrachtungsweisen unumgänglich. Zunehmend forderte die Öffentlichkeit Antworten auf diese Fragen, die sie den Politikern stellte, auf die aber nur der Wissenschaftler eine Lösung frei von Emotionen finden konnte. Der daraus abzuleitenden Verständlichkeit wird Remmert in hohem Maße gerecht. Er betont selbst den Verzicht komplizierter Fachausdrücke und gestattet so, auch allgemeinbiologisch gebildeten Lesern sich einen Einblick in die Ökologie zu verschaffen.

Das vorliegende Lehrbuch enthält eine ausgezeichnete Gesamtdarstellung ökologischer Fragestellungen, Methodologien und Erkenntnisse. Nach einer kurzen aber präzisen Erörterung des Wesens der Ökologie werden ihre Teilgebiete, die Autökologie, die Populationsökologie und die Ökosysteme umfassend besprochen. Im Kapitel Autökologie stellt Remmert ökologische Faktoren wie Salzgehalt, Temperatur, Ernährung, Licht, Sauerstoffangebot, Feuer, zwischenartliche Konkurrenz und Artgenosse als Umweltfaktor vor und untersucht deren Wirkung in eindrucksvoller Weise an zahlreichen Beispielen. Dabei steht im Mittelpunkt der Betrachtung der Selektionswert der ökologischen Parameter und die Angepaßtheit der Organismen. Das Kapitel Populationsökologie beinhaltet die Populationsgenetik, die Demographie, die Verteilung der Organismen im Raum und die Fragen der Populationsdichte. Betont wird die Regulation von Populationen wie Stabilität, Labilität und Mortalität. Letztlich das Kapitel Ökosysteme gibt einen Überblick über „Natürliche" Ökosysteme, den Klimax-Begriff, die Statik und Dynamik in Ökosystemen, die Bedeutung der Tiere im Ökosystem und die veränderlichen und konstanten Ökosysteme. Als besonders informativ sind die jedem Kapitel nachgestellten Fallstudien zu nennen, die die Komplexität ökologischer Vorgänge am Einzelbeispiel belegen.