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Concentrated Gram Stain Smears Prepared with a Cytospin Centrifuge

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A Cytospin slide centrifuge was used to concentrate 0.05- to 0.5-ml samples of cerebrospinal and other body fluids for Gram stain. Trials with cerebrospinal fluid containing known numbers of microorganisms indicated that the Cytospin increased the sensitivity of cerebrospinal fluid Gram stains by up to 2 logs compared with unconcentrated and conventional centrifuge smears. Cytospin-concentrated smears were prospectively compared with unconcentrated Gram-stained smears and bacteriological culture results for 80 clinical body fluid specimens. Bacteria were seen in unconcentrated smears of 9 of the 16 (56%) fluids which were infected, whereas Cytospin smears of 12 of the 16 (75%) showed bacteria. Cytospin smears revealed more bacteria and demonstrated better leukocyte morphology than did unconcentrated or conventionally centrifuged samples of small volumes of infected body fluids, allowing early diagnosis of infection.

Examination of Gram-stained smears of body fluid specimens is an important tool in the rapid diagnosis of infection (9). Since infected body fluids may contain low numbers of microorganisms, use of a concentration technique is recommended (9). Conventional centrifugation is most effective when a large volume of sample is used. In clinical laboratories, however, sample volume is often limited, and preparation of a highly concentrated smear is therefore not possible with conventional techniques.

The Cytospin slide centrifuge is useful for concentration of cells for cytological examination (1, 3, 8, 11) and has been used in one report to aid in the rapid diagnosis and follow-up surveillance of urinary tract infections (10). We used pooled cerebrospinal fluid (CSF) to which bacteria had been added to compare the sensitivity of Cytospin-concentrated, Gram-stained smears with smears of conventionally centrifuged and unconcentrated samples. We also used the Cytospin centrifuge to concentrate 0.05- to 0.5-ml clinical samples of CSF and other body fluids for Gram stain to determine the ability of the Cytospin centrifuge to improve detection of bacteria in small volumes of body fluid specimens when compared with unconcentrated smears.

MATERIALS AND METHODS

Cytospin technique. Each sample was pipetted into a plastic chamber, placed in a Cytospin slide centrifuge (SCA 0030; Shandon Southern Instruments, Inc., Sewickley, Pa.), and forced by centrifugation through a horizontal tube in the chamber, through a hole in a strip of filter paper, and onto a glass slide. The strip of filter paper placed between the plastic chamber and the glass slide absorbed the supernatant fluid, and cells and microorganisms traveled through the hole in the filter paper and were deposited in a 7-mm circular area on the slide. All samples were processed at 2,000 rpm (350 × *g*), the Cytospin's highest speed, since that speed resulted in greater recovery of bacteria than did speeds of 1,000 or 1,500 rpm in preliminary trials. Preliminary trials also showed that thin filter paper strips (SCA 0005; Shandon Southern Instruments) gave superior detection of bacteria when compared with the more absorbent thick strips, and therefore the thin strips were used for the clinical trial. Sterile disposable pipettes (Falcon 7575; Becton, Dickinson & Co., Cockeysville, Md.) were used to deliver 1 drop of fluid to a glass slide for an unconcentrated smear and to introduce samples into the Cytospin chambers, which were filled just before centrifugation. One drop from these pipettes delivered approximately 0.05 ml of fluid. Glass slides were dipped in alcohol and flamed before use. Plastic Cytospin containers were soaked in 3% bleach after use, rinsed with tap water, cleaned thoroughly with an alcohol-soaked cotton swab, and allowed to dry before reuse.

Pooled human serum with bacteria added was used initially to determine optimum conditions for concentration of bacteria in body fluid specimens with a Cytospin slide centrifuge. Sample viscosity was found to significantly influence the quality of the Cytospin smear; therefore, sample volume and duration of centrifugation were adjusted to compensate for those differences in fluids of very high and very low viscosities. Conditions found to provide the highest recovery of bacteria are summarized in Table 1.

Determination of Cytospin smear sensitivity. *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli*

TABLE 1. Cytospin methodology for body fluids

Specimen	Sample vol (drops)	Centrifugation[a] time (min)
High viscosity (e.g., bile, purulent material, synovial fluid)	1	5
Moderate viscosity (e.g., pleural fluid, peritoneal fluid)	5	5
Low viscosity (e.g., CSF, peritoneal dialysate)	10[b]	10

[a] At 2,000 rpm.
[b] 0.5 ml.

(ATCC 25922) were added to pooled CSF to give concentrations of 10^3, 10^4, and 10^5 colony-forming units (CFU) per ml, which were confirmed by plate counts of the samples. These species were chosen because they are isolated frequently and are seen as causative agents of meningitis in our adult patient population. Pooled CSF without added bacteria was also tested as a negative control. Cytospin smears were prepared by centrifuging 10 drops (0.5 ml) of CSF at 2,000 rpm (350 × *g*) for 10 min. Unconcentrated smears were prepared by placing 1 drop of CSF onto a slide and allowing it to dry without further spreading. Conventionally centrifuged smears were prepared by centrifuging 3 ml of sample at 1,000 × *g* for 15 min, removing 2 ml of supernatant fluid, suspending the sediment in the remaining 1 ml of fluid, and applying 1 drop to a slide, where it was allowed to dry without further spreading. The Gram-stained smears were coded and microscopically examined for 2 min by each of three observers, who counted the number of bacteria seen.

Body fluid samples. Eighty body fluid specimens were prospectively studied as received by the microbiology laboratory, under the conditions indicated in Table 1. Gram-stained smears were coded and microscopically examined for 2 min by one observer before culture results were available. Bacteriological cultures were performed and isolates were identified by using standard procedures.

RESULTS

Detection threshold in CSF. Reports of three observers after examining each unconcentrated, conventional centrifuge, and Cytospin smear for 2 min are presented in Table 2. With bacterial concentrations of 10^3 CFU/ml, bacteria were found in 0 of 18 examinations of unconcentrated smears, 0 of 9 examinations of conventional centrifuge smears, and 13 of 18 (72%) examinations of Cytospin smears. With 10^4 CFU/ml, organisms were seen in 7 of 18 (39%) examinations of unconcentrated smears, 7 of 9 (78%) examinations of conventional centrifuge smears, and 18 of 18 examinations of Cytospin smears. Bacteria were found in 100% of the examinations of samples with bacterial concentrations of

TABLE 2. Sensitivities of unconcentrated, conventional centrifuge, and Cytospin-concentrated smears of CSF containing known concentrations of bacteria

Species in sample	Bacterial concn (CFU/ml)	Results								
		Unconcentrated			Conventional centrifuge			Cytospin centrifuge		
		Smear results[a]	Bacteria seen		Smear results[a]	Bacteria seen		Smear results[a]	Bacteria seen	
			Range[b]	Mean[c]		Range[b]	Mean[c]		Range[b]	Mean[c]
S. aureus	10^3	0/6			0/3			4/6	8–20	14
	10^4	4/6	4–22	13	3/3	8–20	12	6/6	8–>100	63
	10^5	6/6	23–>100	99	3/3	15–90	62	6/6	>100	>100
E. coli	10^3	0/6			0/3			3/6	8–19	12
	10^4	3/6	2–4	3	1/3	5	5	6/6	36–>100	82
	10^5	6/6	2–20	8	3/3	11–83	37	6/6	20–>100	>100
S. aureus and *E. coli*[d]	10^3	0/6			0/3			4/6	4–11	6
	10^3	0/6			0/3			5/6	3–11	7
	10^4	0/6			3/3	4–7	5	6/6	2–>100	34
	10^4	0/6			1/3	5	5	6/6	4–25	13
	10^5	5/6	10–>100	46	3/3	25–>100	59	3/3	>100	>100
	10^5	6/6	2–20	7	3/3	7–25	14	3/3	>100	>100

[a] Number positive/number of evaluations.
[b] Number of bacteria seen per positive slide during a 2-minute evaluation.
[c] Total number of bacteria seen divided by number of positive evaluations.
[d] Mixture of *S. aureus* and *E. coli*; results are shown as $\frac{S.\ aureus}{E.\ coli}$. Some positive smears showed only one of the two species present.

TABLE 3. Summary of unconcentrated smear, cytospin smear, and bacteriological culture results in clinical specimens

Specimen type	No. of specimens			
	Examined	Showing organisms on unconcentrated smear	Showing organisms on Cytospin smear	Showing significant growth on culture
Synovial fluid	25	2	2	4
Peritoneal fluid	14	2	3	3
CSF	12	0	0	0
Pleural fluid	7	0	0	1
Bile	5	0	1	1
Interstitial fluid aspirate	5	2	2	2
Wound aspirate	4	3	3	3
Peritoneal dialysate	4	0	0	1
Abdominal aspirate	2	0	1	1
Perinephric fluid	2	0	1	0

10^5 CFU/ml in unconcentrated, conventional centrifuge, and Cytospin smears. Correct reports of the absence of bacteria were given by all three observers for all smears of the CSF pool without added bacteria.

Body fluid results. The types of clinical samples which were evaluated are shown in Table 3. Of the 80 specimens, 16 yielded isolates which were determined by the attending physician or the Infectious Disease Section staff to be true infectious agents. Bacteria were seen in both direct (unconcentrated) and cytospin-prepared smears in 9 of the 16 fluids which were infected. Three additional culture-positive specimens were positive by Gram stain in Cytospin-prepared slides only. The remaining four culture-positive samples were negative on Gram stain by both methods. Organisms seen in Cytospin smears included *S. aureus, Klebsiella pneumoniae, E. coli, Pseudomonas aeruginosa*, enterococcus, streptococci of groups A and B, *Streptococcus intermedius, Corynebacterium* sp., and multiple anaerobes. One sample of perinephric fluid showed 11 budding yeast cells in the Cytospin smear, but no microorganisms were seen in the corresponding unconcentrated smear and no growth occurred on bacteriological culture. However, since a mycology culture was not performed and bacteriological culture plates were discarded after examination at 24 h, with only thioglycolate broth with a tightened cap incubated longer than 24 h, culture conditions were not adequate for the recovery of yeasts.

Although the Cytospin centrifugation conditions chosen were not optimum for the examination of leukocytes, their morphology in Cytospin smears was found to be superior to that in the corresponding unconcentrated smears, facilitating the recognition of intracellular bacteria and enabling ready distinction between polymorphonuclear and mononuclear cells. Gram-positive and gram-negative organisms both exhibited typical staining qualities under these Cytospin centrifugation conditions. Characteristic arrangements of organisms in pairs, chains, or clusters were not distorted by Cytospin centrifugation. Organisms were more readily detected due to their markedly increased numbers in Cytospin smears compared with the corresponding unconcentrated direct smears. Technical problems in preparation of the Cytospin smears were rare. Occasionally, samples would be spread over a wider portion of the slide than the 7-mm circular area, possibly due to poor fitting of the plastic Cytospin chamber against the filter paper and glass slide during centrifugation. When cells in a Cytospin smear were not more numerous than in the corresponding unconcentrated smear, the Cytospin smear was regarded as faulty and a duplicate was prepared.

Subsequent to our prospective study, three recent occurrences in our laboratory point up the utility of Cytospin smears. A small volume of slightly turbid peritoneal dialysis fluid received for routine culture and Gram stain failed to reveal any organisms in an unconcentrated smear, but the Cytospin smear readily demonstrated the presence of gram-negative rods and the culture yielded *P. aeruginosa*. Similarly, an unconcentrated Gram stain of CSF from a patient with a brain abscess and ventriculitis did not demonstrate bacteria, but the Cytospin smear revealed small numbers of gram-negative rods and gram-positive cocci in chains. Counterimmunoelectrophoresis (CIE) for *Streptococcus pneumoniae, Haemophilus influenzae*, and *Neisseria meningitidis* antigens was negative. The CSF lactic acid level was elevated, and a *Limulus* amoebocyte lysate test for endotoxin (Pyrotest; Difco Laboratories, Detroit, Mich.) was positive, suggesting the presence of a gram-negative species. Only a microaerophilic *Strep-*

tococcus sp. was recovered from the specimen, which had not been properly collected or transported for anaerobic culture. A CSF sample from another patient received for routine culture and Gram stain failed to demonstrate organisms in the unconcentrated smear, but a single large budding yeast cell was seen in the Cytospin smear. The latex agglutination test for cryptococcal antigen was negative, but *Cryptococcus neoformans* was recovered from the culture.

DISCUSSION

Bacteriological culture is the most sensitive single means of detecting the specific pathogen in bacterial meningitis (2). A sensitive, rapid diagnostic aid is desirable. Concentrations of bacteria ranging from 10^2 to 10^9 CFU/ml have been reported in the CSFs of patients with meningitis (5, 7). We found that bacteria were reliably detected on unconcentrated Gram-stained smears of CSF samples containing 10^5 *E. coli* and *S. aureus* CFU/ml, but were less likely to be found on unconcentrated or conventional centrifuge smears of samples containing bacterial concentrations below that level. This is consistent with the findings of Feldman (4), who, in examining CSF samples from infants and children with meningitis, reported that bacteria were seen on direct smears of all of 40 specimens having bacterial concentrations above 10^5 CFU/ml, but on only 3 of 10 samples having bacterial concentrations below that level. Fossieck and Fedorko (6) also reported a similar sensitivity of direct Gram stains in detecting organisms in blood culture media.

Several types of concentration techniques may be useful in preparing smears for staining. Multiple drops of CSF might be layered onto the same area of a slide, allowing each drop to dry before the next is applied. Conventional centrifugation of even small volumes of fluid will also sometimes be profitable. Although our results in Table 2 indicate approximately equal (or sometimes fewer) numbers of organisms in the conventionally concentrated smear compared with an unconcentrated one, only a threefold concentration was attempted, since clinical microbiology laboratories usually receive less than 3 ml of CSF for processing. Longer centrifugation and increased centrifugal force would be expected to increase the number of bacteria seen. A prolonged examination of any smear would also increase the number of organisms found. We do not recommend limiting examinations of smears from clinical specimens to only 2 min. A short, uniform period for examination was used for our comparison only to determine how readily microorganisms could be found under the various conditions. The up to 2-log increase in sensitivity that we achieved with the Cytospin on 0.5-ml specimens is comparable to the concentration of 100 ml of CSF to a volume of 1.0 ml by conventional techniques. Although the Cytospin does not concentrate material for culture as conventional centrifugation does, it produces a highly concentrated smear and provides sensitive, rapid information while growth of the more sensitive culture is being awaited.

CIE for detection of capsular polysaccharide antigens is another tool used in the rapid diagnosis of meningitis. Feldman (4) found that of CSF samples from five meningitis patients which showed no detectable antigen by CIE, only one specimen had a bacterial concentration above 10^3 CFU/ml. Fung and Wicher (7) reported that the minimum concentration of *S. pneumoniae* or *H. influenzae* detected by CIE was 10^3 CFU/ml and that the minimum detectable group B *Streptococcus* concentration was 10^6 CFU/ml in the blood of animals, although consistently positive results required concentrations at least 2 logs higher. These findings suggest that the sensitivity of Cytospin smears of 0.5-ml CSF samples may be qualitatively equivalent to that of CIE as a rapid diagnostic aid. Cytospin smears offer the advantages of availability and simplicity, with no need for antisera (which may be expensive and difficult to obtain), no need for training to perform the relatively complicated CIE procedure, and no restriction to detection of organisms for which specific antisera are available. As an adjunct to CIE, Cytospin smears should aid in confirmation and interpretation of CIE findings, since some serological cross-reactivity has been observed (2).

The use of a slide centrifuge for concentration of potentially infectious material may raise questions regarding safety of the procedure, but sample volume is small and concentrations of bacteria in infected fluids are often low. The Cytospin has been used by hematology laboratories in the United States for more than 15 years, and to our knowledge its use has not been associated with outbreaks of infectious disease. Although we feel that the risks of this procedure are minimal, if further precautions are desired, sealed chambers, which can be used to enclose the entire apparatus for each sample separately and prevent the dispersion of any aerosols created during centrifugation, are available from the manufacturer.

We have found the Cytospin centrifuge to be effective in increasing the number of specimens of various body fluids in which microorganisms can be rapidly detected and in increasing the number of bacteria found per smear over the number seen on unconcentrated smears. In addition to the quantitative benefits, we also found an improvement in the quality of the smears. The Cytospin centrifuge provides a simple, rap-

id, and effective means of concentrating CSF and other body fluid specimens for Gram stain, even when 0.5 ml or less of specimen is available; as a useful supplement to conventional procedures, it aids in the early diagnosis of infection.

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