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NOTES

Test Characteristics of Acridine Orange, Gram, and May-Grünwald-Giemsa Stains for Enumeration of Intracellular Organisms in Bronchoalveolar Lavage Fluid

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For enumeration of intracellular organisms (ICO) in bronchoalveolar lavage fluid samples, the May-Grünwald-Giemsa (MGG) stain displayed higher interobserver agreement than the acridine orange and Gram stains. The MGG stain offered a reliable enumeration of ICO when 200 cells were counted by one observer.

The microscopic enumeration of intracellular organisms (ICO) present in bronchoalveolar lavage (BAL) cells has been described as a diagnostic tool in the differentiation between ventilator-associated pneumonia (VAP) and airway colonization (1, 3, 4, 7, 9, 10, 13, 15). However, the various studies on ICO differ with respect to the stain used and the number of cells counted. Moreover, to the best of our knowledge, the test characteristics of the different stains in this setting have not yet been evaluated. Therefore, we decided to investigate the reproducibilities and interobserver agreements of the acridine orange (AO), Gram, and May-Grünwald-Giemsa (MGG) stains for enumeration of ICO in BAL fluid samples and to determine the minimal number of cells that must be counted for a reliable enumeration of ICO.

Seventy-seven BAL fluid samples were obtained from 56 patients with suspected VAP. The total cell count was performed in a Fuchs-Rosenthal hemocytometer. Cytocentrifugation was performed with the Cytospin 3 (Shandon Scientific Ltd., Astmoor, England), at a speed of 650 rpm (approximately $40 \times g$) for 10 min, at the low acceleration rate. The number of drops per preparation was adjusted according to the total cell count (2). Preparations were stained with Gram and MGG stains, sealed (xylene substitute mountant; Shandon Scientific Ltd.), and stored at room temperature. A third preparation was stored at −30°C and stained with the AO stain on the day of examination. Gram staining was performed according to the conventional method (8) by using crystal violet (Merck [Darmstadt, Germany] 1408), fuchsin (Merck 15937), potassium io-

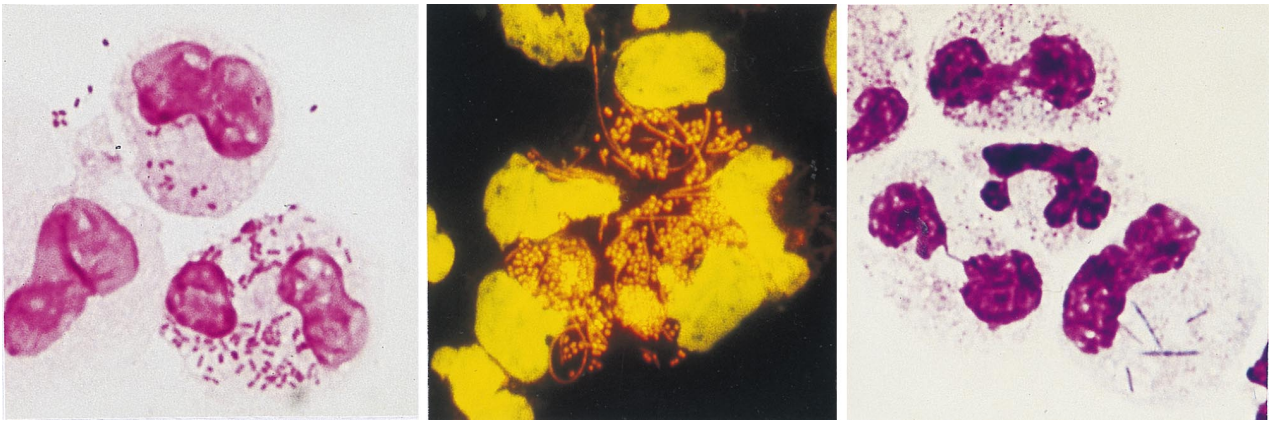

FIG. 1. Intracellular organisms in cytocentrifuged BAL fluid specimens. (Left) Gram-stained coccobacilli. (Center) AO-stained mixed flora in aspiration pneumonia. (Right) MGG-stained filamentous rods.

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TABLE 1. Random-factor ANOVA results and estimates of variance components for the MGG, AO, and Gram stains in counting ICO in 50 cytocentrifuged BAL fluid samples

Stain	Source[a]	Sums of squares	df	Mean sums of squares	Expected mean sums of squares[b] (SE)	Relative importance of source of variation (%)
MGG	S	73,636.21	76	968.90	95.94 (15.52)	94
	O	0.13	1	0.13	0.00 (0.02)	0
	C	4.48	4	1.12	0.00 (0.04)	0
	SO component	721.87	76	9.50	0.89 (0.32)	1
	SC component	1,402.13	304	4.61	0.00 (0.28)	0
	OC component	38.87	4	9.72	0.06 (0.07)	0
	SCO component	1,534.13	304	5.05	5.05 (0.41)	5
	Total	77,337.82	769		101.94	100
AO	S	113,963.92	76	1,499.53	144.96 (24.03)	90
	O	260.66	1	260.66	0.55 (0.55)	0
	C	32.41	4	8.10	0.01 (0.04)	0
	SO component	3,790.95	76	49.88	8.64 (1.60)	6
	SC component	1,951.59	304	6.42	0.00 (0.37)	0
	OC component	25.09	4	6.27	0.00 (0.05)	0
	SCO component	2,029.31	304	6.68	6.68 (0.54)	4
	Total	122,053.93	769		160.84	100
Gram	S	70,122.61	76	922.67	89.40 (14.78)	86
	O	0.06	1	0.06	0.00 (0.02)	0
	C	70.49	4	17.62	0.03 (0.07)	0
	SO component	1,912.44	76	25.16	3.15 (0.82)	3
	SC component	3,930.71	304	12.93	1.75 (0.65)	2
	OC component	28.59	4	7.15	0.00 (0.06)	0
	SCO component	2,869.41	304	9.44	9.44 (0.76)	9
	Total	78,934.31	769		103.77	100

[a] S, specimen; O, observer; C, number of cells counted. Components are combinations of these sources of variation. The SCO component includes residual error.
[b] Negative estimates are replaced by zeros.

dide (Merck 5043), and resublimed iodine (Merck 4761). MGG staining was carried out with May-Grünwald's eosin-methylene blue solution (Merck 1424) and Giemsa solution (Merck 9204) (6). The AO stain was purchased from Difco (Detroit, Mich.; catalog no. 3336-75-9), and staining was performed according to the instructions of the manufacturer.

The Gram-, MGG-, and AO-stained preparations were examined by two observers in a double-blinded fashion at a magnification of ×1,000 by using oil immersion. For the AO stain, a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with a filter set 09 487909-0000 (excitation, 450 to 490 nm; emission, 520 nm) was used. For each stain, the number of cells with ICO was recorded after 100, 200, 300, 400, and 500 cells were counted and was expressed as a percentage of the number of nucleated cells counted.

Testing for differences in counting among staining methods was done within a mixed-model analysis-of-variance (ANOVA) design by taking repeated measurements with varying numbers of observers and varying numbers (in hundreds) of cells counted. Variance components to be used in calculating intraclass correlation coefficients for reproducibility and interobserver agreement were estimated; for convenience, these coefficients will be referred to below as reproducibility and interobserver agreement, respectively. Formulas are based upon G. R. Norman's quasiclassical R measurements, which are very closely related to the ρ^2 measurements in generalizability theory (or G theory) (12, 14). In addition to these, the "overall" ϕ value from G theory, which can be seen as a combined reproducibility and agreement measurement (11), was used. A ϕ value of ≥ 0.95 was considered acceptable. Finally, a decision study (or D study) was performed on the ϕ value to investigate how many hundreds of cells must be counted by how many observers in order to obtain a ϕ value of ≥ 0.95. All data were analyzed by SPSS-PC, version 6.1.3, and by generalized analysis of variance (GENOVA) (5).

ICO were demonstrated in 50 of 77 cytocentrifuged BAL fluid preparations (Fig. 1). The differences among the three stains in numbers of ICO counted were not statistically significant (quasi-F ratio = 1.29 by 2 and 6 df; $P > 0.25$).

Table 1 shows the two-way repeated-measurements ANOVA results and the estimates of the variance components for the three staining methods. The reproducibility of the Gram stain was lower than those of the AO and MGG stains (Table 2), and the MGG stain displayed the highest interobserver agreement (Table 3). For the MGG stain, 94% of variations in counting were attributable to the specimens (S), as can be seen in the last column of Table 1. This represented the "natural" variation in numbers of ICO among the study samples. Only 1% of all variance was due to the fact that observers (O) differed in counting the specimens (SO component), but

TABLE 2. Intraclass correlation coefficients for reproducibility of the three staining methods[a]

10^2 Cells counted	Correlation coefficient for:		
	MGG stain	AO stain	Gram stain
1	0.951	0.959	0.892
2	0.975	0.979	0.943
3	0.983	0.986	0.961
4	0.987	0.989	0.971
5[b]	0.990	0.991	0.976

[a] The number of cells counted was varied, and one observer was used. Correlation coefficients were calculated by D analysis, except where indicated otherwise.
[b] Results calculated according to G theory.

TABLE 3. Intraclass correlation coefficients for interobserver agreement of the three staining methods[a]

No. of observers	Correlation coefficient for:		
	MGG stain	AO stain	Gram stain
1	0.942	0.904	0.879
2[b]	0.966	0.924	0.935
3	0.974	0.930	0.956

[a] One hundred cells were counted, and the number of observers was varied. Results were calculated by D analysis, except where indicated otherwise.
[b] Results were calculated according to G theory.

there were no estimated differences in counting among the varying numbers (in hundreds) of cells (C) counted (SC component). Because the variations due to interaction among the variations in the specimens, number of cells counted, and observers (the SCO component, which can be regarded as the residual error component) were also low (5%), both reproducibility and interobserver agreement for the MGG stain were high. For the AO stain, differences among observers accounted for 6% of all variations, resulting in lower interobserver agreement. The influence of antimicrobial agents on the bacteria partly explained the differences among observers. Bacteria on three BAL fluid preparations obtained from patients who were given antimicrobial agents at the time of bronchoscopy appeared as faint green silhouettes, which were consistently reported as ICO by only one observer. Further evaluation of this phenomenon revealed that, when subjected to subinhibitory concentrations of antimicrobial agents, some bacteria fluoresced while others did not. In addition, on AO-stained preparations, cell borders were not always clearly discernible, making distinction between ICO and extracellular organisms difficult. Regarding the Gram stain, the differences among observers and the differences among the numbers of cells counted formed a relatively large part of the variations (3 and 2%, respectively). The residual error was 9%. These factors were responsible for the relatively low interobserver agreement with the Gram stain. It is tempting to speculate that the presence of erythrocytes and intercellular debris interfered with the recognition of ICO.

Both the MGG and Gram staining methods reached the acceptable ϕ value of 0.95 when a single observer counted the ICO. With the MGG stain, the ϕ value was reached when 200 cells were counted, while the Gram staining method reached this value when 700 cells were counted. With the AO stain, however (due to its lower interobserver agreement), two observers, each counting 200 cells, were needed to reach the acceptable ϕ value.

In conclusion, this study demonstrated that for enumeration of ICO in BAL fluid samples, the MGG stain was superior to the Gram and AO stains. The MGG stain displayed the best interobserver agreement and allowed for a reliable enumeration of ICO by one observer counting 200 cells.

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