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Pneumocystis carinii pneumonia in HIV-infected patients: diagnostic yield of induced sputum and immunofluorescent stain with monoclonal antibodies

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Pneumocystis carinii *pneumonia in HIV-infected patients: diagnostic yield of induced sputum and immunofluorescent stain with monoclonal antibodies. J. Fortún, E. Navas, P. Martí-Belda, P. Montilla, J.M. Hermida, M.J. Pérez-Elías, L. Buzón, A. Guerrero.*
ABSTRACT: The purpose of this study was to evaluate the diagnostic yield of induced sputum (IS), assessing the reliability of indirect immunofluorescent stain with monoclonal antibodies (IFMoAb) and methenamine silver (Met-Ag) and analysing factors likely to influence the sensitivity of these techniques.

An analysis was prospectively carried out on IS specimens collected from 61 human immunodeficiency virus (HIV)-infected patients during 69 episodes of suspected *Pneumocystis carinii* pneumonia.

Ultrasonic nebulizers with hypertonic 2% saline were used. IFMoAb to *P. carinii* and Met-Ag were performed after cytocentrifugation of the specimen. Results were compared with those of bronchoalveolar lavage (BAL) with/without transbronchial biopsy (TBB), performed not more than seven days after induction of sputum.

***P. carinii* pneumonia was confirmed in 32 episodes, of which IS was diagnostic in 23. The sensitivity of the staining procedures was 69% for IFMoAb, and 28% for Met-Ag. The three episodes of *P. carinii* pneumonia in patients on oral chemoprophylaxis yielded negative IS results; in contrast, IS was negative in only 6 of the 29 cases not receiving chemoprophylaxis.**

IS is a non-aggressive procedure that diagnosed *P. carinii* pneumonia in 72% of our cases. The yield increased significantly when IFMoAb was used in patients not receiving oral chemoprophylaxis.
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The alveolar location of *P. carinii* implies that diagnostic procedures must ideally obtain respiratory samples representative of that area. Inhalation of hypertonic saline is an easy and bloodless method of obtaining sputum ("induced sputum" (IS)) representative of the bronchoalveolar tract, in many cases removing the need to perform bronchoalveolar lavage (BAL) or a transbronchial biopsy (TBB).

The results of IS examination in the diagnosis of *P. carinii* pneumonia are good [1–6], especially if the staining procedure includes monoclonal antibody to *P. carinii* [3–5]. Several monoclonal antibody techniques have been developed: immunofluorescence [3, 5, 7–10], immunoperoxidase [4] and enzyme-linked immunoadsorbent assay (ELISA) [11].

Our study had three aims: to evaluate the diagnostic yield of IS in our patients, to assess the reliability of two staining techniques (indirect immunofluorescent stain with monoclonal antibodies (IFMoAb) and methenamine silver (Met-Ag)), and to analyse factors influencing the sensitivity of these techniques. With this intention we carried out a prospective study of the usefulness of IS in human immunodeficiency virus (HIV) infected patients with suspected *P. carinii* pneumonia.

Material and methods

Patient selection. From March 1989 to April 1990 we made a prospective analysis of all HIV-infected patients hospitalized in our unit with clinical presentation suggestive of *P. carinii* pneumonia.

Clinical and analytical evaluation. From the medical background of the patients the following data were noted: the presence of adult immune deficiency syndrome (AIDS) diagnostic criteria (according to the revised case surveillance definition of AIDS, Center for Diseases Control (CDC), September 1987 [12]), the existence of a previous episode of *P. carinii* pneumonia, and chemoprophylaxis (primary or secondary) of *P. carinii* during at least the preceding month.

The diagnosis of HIV-infection was made by ELISA (two different positive determinations), with Western-blot confirmation in most cases.

Sputum induction. This was performed in separate rooms, mostly after fasting, and following brushing of the teeth and antiseptic mouth rinsing. Ultrasonic nebulizers were used (DeVilbiss, Ultra-Neb 99TM; Mistagen, model EN 143-A), with hypertonic 2% saline. Induction duration was 15–20 min, obtaining one specimen (2–5 ml) per patient.

Sputum processing. Most specimens were processed immediately after collection. In some cases the sputum was refrigerated at 4°C and processed 24 h later. In all cases the following techniques were used: IFMoAb to *P. carinii*, Met-Ag rapid stain, direct immunofluorescent stain and Legionella culture, auramine-rhodamine stain, mycobacterial culture, Gram's stain and aerobic culture when the Gram's stain revealed more than 25 polymorphonuclear neutrophils (PMNs) per field. Fungal cultures were not performed routinely. IFMoAb to *P. carinii* was carried out after cytocentrifugation of the specimen, according to the manufacturer's instructions (Pneumocystis-Direkt-Nachweis-test, Progen Biotechnik Gmbh; Im Neuenheimer Feld 519, 6900 Heidelberg, West Germany); 3% sodium lauryl sulphate was used as the mucolytic agent. The Met-Ag was also carried out after cytocentrifugation. All slides were reviewed and confirmed by two different observers.

Fibrobronchoscopy. Fibrobronchoscopy (FB) was performed not more than seven days after induction of sputum. BAL was carried out after wedging the bronchoscope in the middle lobe, instilling 150 ml of saline in 50 ml aliquots. Blind TBB was performed after lavage. BAL specimens were cytocentrifugated and stained with IFMoAb, Met-Ag, Papanicolaou, May-Grünweld-Giemsa and periodic acid-Schiff (PAS). TBB were processed following conventional histological techniques.

P. carinii *infection criteria*

Definite. Definite *P. carinii* infection was considered when more than five cysts per slide were seen in respiratory secretions (IS, BAL or TBB), together with compatible clinical presentation. When IS was positive but FB was not performed, definite *P. carinii* infection was considered if the patient fulfilled the above characteristics, no other aetiological agents were apparent and an adequate clinical response to pentamidine or co-trimoxazole was observed.

Probable. When IS was negative and FB was not performed, probable *P. carinii* infection was considered when clinical presentation was compatible, X-ray findings were either diffuse bilateral mixed alveolar-interstitial infiltrates or clear lungs, lactate dehydrogenase (LDH) was >1.7 $\mu mol \cdot s^{-1} \times l$ [>500 IU], total CD4 lymphocytes $<0.2 \times 10^9 \cdot l^{-1}$ [$<200 \cdot mm^{-3}$], no other microorganism was found during admission or in the first two months of follow-up, and response to pentamidine or co-trimoxazole was seen in terms of clinical improvement, improvement of arterial blood gases and reduction in LDH.

Statistical analysis. The statistical significance was calculated by the Chi-squared test, using the Yate's correction for small samples. A p value of <0.05 was considered significant.

The sensitivity of the diagnostic tests was calculated by dividing the number of positive cases for each test (×1,000) by the total number of positive cases.

The confidence intervals (CI) for a $p<0.05$ were calculated using the formula:

$$p \pm 1.96\sqrt{\frac{p(1-p)}{n}}$$

Results

A study was made of 69 episodes clinically suggestive of *P. carinii* infection, occurring in 61 patients.

All confirmed *P. carinii* pneumonia cases are registered in table 1. FB was performed in 24 cases; *P. carinii* was identified in 20 cases and *M. tuberculosis* in four. IS examination was positive in 12 of these cases (60%), 11 with IFMoAb staining and 3 with Met-Ag (p=0.026).

In the remaining 45 episodes, IS was the only diagnostic procedure used, being diagnostic in 11 cases; IFMoAb staining was positive in 11 and Met-Ag in 6. All cases fulfilled the above-mentioned criteria of definite *P. carinii* infection.

An analysis of the remaining 34 episodes showed only one case fulfilling the criteria of probable *P. carinii* pneumonia; IS in this patient had been obtained 8 days after empirical co-trimoxazole therapy was begun.

Of the 33 remaining episodes, a second pathogen potentially responsible for the clinical picture was isolated in 29, whilst in the remaining 4 cases the symptoms cleared up without therapy.

In brief (table 2), IS examination was diagnostic in 23 out of 32 (20+11+1) pneumonias due to *P. carinii* (sensitivity 72%; CI: 0.57–0.87). IFMoAb was positive in 22 (69%) and Met-Ag in 9 (28%) (p=0.002).

In spite of the low CD4 lymphocyte count, and possibly because in most cases *P. carinii* infection was the first manifestation of HIV infection, only three patients were receiving oral chemoprophylaxis (two secondary, one primary). The three patients (two receiving Fansidar and one co-trimoxazole) had negative IS examination; in contrast, of 29 patients not receiving chemoprophylaxis, only six had negative IS (table 3), (p<0.05).

In all cases the sputum induction was well-tolerated, and no secondary effects were noted.

Table 1. – Results of the diagnostic procedures used in 32 episodes of *P. carinii*

Pt no.	Fibrobronchoscopy			Induced sputum	
	BAL		TBB		
	IFMoAb	Cytology		IFMoAb	Met-Ag
1	-	NP	+	-	-
2	+	+	NP	-	-
3	-	+	NP	-	+
4	NP	+	+	+	-
5	+	+	+	+	-
6	+	+	+	+	-
7	+	+	+	-	-
8	+	+	+	-	-
9	+	+	+	+	+
10	+	+	+	-	-
11	+	-	NP	+	+
12	+	+	+	-	-
13	-	+	NP	-	-
14	+	+	+	+	-
15	+	+	+	+	-
16	+	+	+	+	-
17	NP	+	NP	-	-
18	NP	+	+	+	-
19	+	NP	+	+	-
20	+	NP	NP	+	-
21	NP	NP	NP	+	-
22	NP	NP	NP	+	+
23	NP	NP	NP	+	+
24	NP	NP	NP	+	-
25	NP	NP	NP	+	+
26	NP	NP	NP	+	-
27	NP	NP	NP	+	+
28	NP	NP	NP	+	-
29	NP	NP	NP	+	+
30	NP	NP	NP	+	+
31	NP	NP	NP	+	-
32	NP	NP	NP	-	-
Total*	17	17	14	32	32
+ve	14	16	14	22	9

NP: not performed; *: total performed; +ve: positive; BAL: bronchoalveolar lavage; TBB: transbronchial biopsy; IFMoAb: immunofluorescence stain with monoclonal antibodies; Met-Ag: methenamine silver.

Table 2. – Sensitivity of induced sputum in diagnosis of *P. carinii* pneumonia

Number of episodes	69
P. carinii pneumonia	32
Definite	31
Probable	1
Positive induced sputum	23
IFMoAb	22 (69)*
Met-Ag	9 (28)*
IFMoAb + Met-Ag	8
No *P. carinii* infection	37
Response without therapy	4
Other aetiological agents	33

(): % of *P. carinii* cases; *: p=0.002. For abbreviations see legend to table 1.

Table 3. – Chemoprophylaxis/induced sputum (IS) relationship in patients with *P. carinii* pneumonia

	Patients receiving chemoprophylaxis	Patients not receiving chemoprophylaxis
IS positive	0*	23*
IS negative	3	6

*: $p<0.05$. See text.

Discussion

BAL, with or without TBB, enables diagnosis of *P. carinii* pneumonia in 85–100% of cases depending on the series [13–15]. Unfortunately, these rather aggressive procedures require the participation of trained staff, they are expensive and time-consuming. Successful results obtained by examination of spontaneously expectorated sputum amount to only 16% in HIV infected patients [16] and 6% in patients with other kinds of immunodeficiency [17, 18]. The use of ultrasonic nebulizers provides samples representative of the bronchoalveolar tract, clearly increasing sputum sensitivity in AIDS patients [1–6], as well as in other immunosuppressed states [19], and is easy and cheap.

In our series, IS examination was diagnostic in 72% of cases, with a confidence interval of 57–87%. Most of the results of other trials are in this range. This high yield from a non-aggressive procedure has currently made IS the first diagnostic step in HIV-infected patients with suspected *P. carinii* pneumonia.

Initial experiences with IS were performed with conventional processing techniques for *P. carinii* visualization (Met-Ag, toluidine blue, Giemsa stain...) (table 4). Recently, monoclonal antibody-based stains, the effectiveness of which is similar for the previously mentioned techniques in BAL examination [7–10], have shown a higher sensitivity than conventional stains in samples obtained by IS [3–5]. In the series of MIDGLEY *et al.* [3], 15 out of 47 (31%) patients diagnosed by IS were positive by means of IFMoAb stain and negative by Met-Ag. Similarly, the study of KOVACS *et al.* [5] demonstrated a better yield by means of IFMoAb in comparison with Diff-Quick (a modified Giemsa stain) with near statistical significance (p=0.07). Of the series displayed in table 4, the only investigator with similar results with either technique used immunoperoxidase instead of immunofluorescence [4]. Our work confirms the superiority of IFMoAb stains over Met-Ag, with statistical significance. The poor results obtained with Met-Ag cannot be attributed to bad technical performance, as the experience of the staff responsible has been proved in previous reports, which confirmed a Met-Ag stain sensitivity in BAL samples of up to 95% [20]. Although the study design does not permit IFMoAb stain specificity to be calculated, trials carried out by other investigators confirm the near lack of false positives [7], especially when only samples with more than five cysts per smear are considered diagnostic.

Table 4. – Yield of induced sputum in available literature

First author [Ref.]	Classical techniques No. pts*	(%)		MoAb No. pts*	(%)	
PITCHENIK [1]	11/20	(55)	SM	-		
BIGBY [2]	14/25	(56)	DQ	-		
MIDGLEY [3]	32/64	(50)	SM	47/64	(73)	IF
BLUMENFELD [4]	12/18	(67)	DQ	12/18	(67)	IP
KOVACS [5]	39/49	(80)	TB	45/49	(92)	IF
KOVACS [5]	37/49	(76)	DQ	45/49	(92)	IF
LEIGH [6]	18/19	(94)	SM	-		

*: positive induced sputum/total number of *P. carinii*; MoAb: monoclonal antibody; SM: silver methenamine; DQ: Diff-Quick; TB: toluidine blue; IF: immunofluorescence; IP: immunoperoxidase.

Therefore, we think that increased sensitivity without loss of specificity makes IFMoAb one of the best indicated techniques in processing samples obtained by IS in *P. carinii* pneumonia diagnosis.

Sputum induction did not produce any adverse effect in our patients. NELSON *et al.* [21], report a high morbidity and mortality in patients with pleural effusion, especially in those presenting pulmonary Kaposi's sarcoma. In our series there were no patients with pleural effusion and the only two with Kaposi's sarcoma lacked visceral extension.

For comparison, our protocol included, as the "gold standard" test performed in all patients, a procedure of proven high sensitivity such as BAL [13–15], with or without TBB. As the study underway demonstrated a high probability of diagnosis, coupled with problems in performing FB (important respiratory compromise, patient refusal or technical difficulties) FB was reserved for those cases with high clinical suspicion but negative IS examination. Therefore, analysis of FB sensitivity in those patients with simultaneous IS examination shows a low sensitivity (60%) in comparison with the real one (72%), due to this sample bias.

Of the patients diagnosed by IS, none were receiving *P. carinii* chemoprophylaxis. However, three of the nine patients with negative IS were ($p<0.05$) (table 3). JULES-ELYSEE *et al.* [22] have recently demonstrated a diminished BAL sensitivity for *P. carinii* pneumonia diagnosis in HIV-infected patients receiving inhaled pentamidine prophylaxis (62 *versus* 100% in the control population; $p<0.05$). Our data demonstrate that, at least concerning IS, oral chemoprophylaxis (none of our patients received inhaled pentamidine) also significantly decreased its diagnostic yield. Unfortunately, the limited number of cases does not permit us to reach conclusions with regard to BAL sensitivity in these patients. In two of the three patients who received chemoprophylaxis, FB was performed, BAL being positive in one (TBB was positive in both). The three patients had typical diffuse interstitial lung infiltrates.

We conclude that IS examination in *P. carinii* pneumonia diagnosis is a non-aggressive, easy to perform, technique with a high diagnostic yield. In our study, 72% of all cases were diagnosed in this way. Sensitivity increases significantly if IFMoAb are used in the processing. Its performance is highly recommended in all patients with suspected *P. carinii* pneumonia, excluding those receiving chemoprophylaxis, in which its yield notably decreases.

References

1. Pitchenik AE, Gankei P, Torres A, Evans DA, Rubin E, Baier H. – Sputum examination for the diagnosis of *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome. *Am Rev Respir Dis*, 1986; 133: 226–229.
2. Bigby TD, Margolskee D, Curtis JL, Michael PF, Sheppard D, Hadley WK, Hopewell PC. – The usefulness of induced sputum in the diagnosis of *Pneumocystis carinii* pneumonia in patients with the acquired immunodeficiency syndrome. *Am Rev Respir Dis*, 1986; 133: 515–518.
3. Midgley J, Leigh TR, Shanson DC, Parsons A, *et al.* – Increased sensitivity of immunofluorescence for detection of *P. carinii*. *Lancet*, 1989; 23(30): 1523.
4. Blumenfeld W, Kovacs JA. – Use of monoclonal antibody to detect *Pneumocystis carinii* in induced sputum and bronchoalveolar lavage fluid by immunoperoxidase staining. *Arch Pathol Lab Med*, 1988; 112: 1233–1236.
5. Kovacs JA, Ng VL, Masur H, Leoung G, Hadley WK, Evans G, *et al.* – Diagnosis of *Pneumocystis carinii* pneumonia: improved detection in sputum with use of monoclonal antibodies. *N Engl J Med*, 1988; 318(10): 589–593.
6. Leigh TR, Hume C, Gazzard B, Parsons P, Husain OAN, Collins JV. – Sputum induction for diagnosis of *Pneumocystis carinii* pneumonia. *Lancet*, 1989; July 22: 205–207.
7. Kovacs JA, Swan JC, Shelhamer J, Gill V, Ognibene F, Parrillo JE. – Prospective evaluation of a monoclonal antibody in diagnosis of *Pneumocystis carinii* pneumonia. *Lancet*, 1986; July 5: 1–3.
8. Milder JE, Walzer PD, Coonrod JD, Tutledge ME. – Comparison of histological and immunological techniques for detection of *Pneumocystis carinii* pneumonia in rat bronchial lavage fluid. *J Clin Microbiol*, 1980; 11(4): 409–417.
9. Gill VJ, Evans G, Stock F, Parrillo JE, *et al.* – Detection of *P. carinii* by fluorescent-antibody stain using a combination of three monoclonal antibodies. *J Clin Microbiol*, 1987; 25(10): 1837–1840.
10. Dournon E, Rajagopalan P, Albert F, Camus F, *et al.* – Diagnosis of *P. carinii* pneumonia by non-experts. *Lancet*, 1989; Jan. 14: 107–108.
11. Linder E, Lundin L, Vorma H. – Detection of *Pneumocystis carinii* in lung-derived samples using monoclonal antibodies to an 82 kDa parasite component. *J Immunol Meth*, 1986; JIM04270.
12. Center for Diseases Control. – Revision of the CDC surveillance case definition for acquired immunodeficiency syndrome. *MMWR*, 1987; 36(Suppl.): 1.
13. Broaddus C, Dake MD, Stulberg MS, Brumenfeld W. – Bronchoalveolar lavage and transbronchial biopsy for the diagnosis of pulmonary infections in the AIDS. *Ann Intern Med*, 1985; 102: 747–752.
14. Stover DE, White DA, Romano PA, Gellene RA, *et al.* – Diagnosis of pulmonary disease in AIDS. Role of bronchoscopy and bronchoalveolar lavage. *Am Rev Respir Dis*, 1984; 130: 659–662.

15. Mills J. – *Pneumocystis carinii* and *Toxoplasma gondii* infections in patients with AIDS. *Rev Infect Dis*, 1986; 8(6): 1001–1011.
16. Del Río C, Guarner J, Honig EG, Slade BA. – Sputum examination in the diagnosis of *Pneumocystis carinii* pneumonia in the AIDS. *Arch Pathol Lab Med*, 1988; 112: 1229–1232.
17. Walzer PD, Perl DP, Krogstad DJ, Eawson PG, Schulz MG. – *Pneumocystis carinii* pneumonia in the United States: epidemiologic, diagnostic and clinical features. *Ann Intern Med*, 1974; 80: 83–93.
18. Lau WK, Young LS, Remington JS. – *Pneumocystis carinii* pneumonia: diagnosis by examination of pulmonary secretions. *J Am Med Assoc*, 1976; 236: 2399–2402.
19. Masur H, Gill VJ, Ognibene FP, Shelhamer J, Godwin C, Kovacs JA. – Diagnosis of *Pneumocystis carinii* pneumonia by induced sputum technique in patients without the acquired immunodeficiency syndrome. *Ann Intern Med*, 1988; 109: 755–756.
20. Fortún J, Hermida JH, Hernández-Cabrero JM, Martí-Belda P, Sueiro A, Fogué L, Sanz I, Buzón L. – Neumonía por *Pneumocystis carinii* en pacientes con infección por VIH en un hospital español. *Enf Infec Microbiol Clin*, 1990; 8(2): 82–87.
21. Nelson M, Bower M, Smith D, Gazzard BG. – Life-threatening complication of sputum induction. *Lancet*, 1990; i: 112–113.
22. Jules-Elysee KM, Stover DE, Zaman MB, Bernard EM, White DA. – Aerosolized pentamidine: effect on diagnosis and presentation of *P. carinii* pneumonia. *Ann Intern Med*, 1990; 112: 750–757.