Direct immuno-fluorescence for the diagnosis of legionellosis

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DJM HALDANE, R PEPPARD, RK SUMARAH. Direct immunofluorescence for the diagnosis of legionellosis. Can J Infect Dis 1993;4(2):101-104. Culture and direct immunofluorescent microscopy (DFA) results for *Legionella pneumophila* were reviewed over a two-year period. In the first year, a positive result was defined as having at least one morphologically typical fluorescing organism. In the second year, a positive was defined as at least five typical fluorescing organisms. Despite these stricter criteria and other measures to reduce the possibility of reagent contamination, there was no statistically significant difference in the sensitivity or specificity of the DFA in the two years for sputa, deep specimens or overall. Of 37 sputum specimens from infected patients, 16 were positive on DFA. Thirty-two of 38 positive patients were detected by sputum culture. DFA can provide rapid diagnostic information but cannot be used to rule out the diagnosis. Sputum is a useful specimen for the initial laboratory investigation of patients with legionellosis.

Key Words: Immunofluorescence, *Legionella*, Sensitivity, Specificity

L'immunofluorescence directe dans le diagnostic de la légionellose

RÉSUMÉ: Les résultats de culture et de microscopie par immunofluorescence directe (DFA) concernant *Legionella pneumophila* ont été passés en revue sur une période de deux ans. Pour la première année, un résultat positif a été défini par au moins un micro-organisme fluorescent morphologiquement typique. Dans la deuxième année, un résultat positif a été défini par au moins 5 micro-organismes fluorescents morphologiquement typiques. Malgré ces critères plus stricts et d'autres mesures visant à réduire la possibilité d'une contamination des réactifs, il n'a pas été observé de différence statistiquement significative dans la sensibilité ou spécificité de la microscopie en DFA au cours des deux ans, concernant les expectorations, les prélèvements profonds ou l'ensemble des prélèvements. Sur 37 échantillons d'expectoration provenant de patients infectés, 16 étaient positifs à l'examen microscopique en DFA. Trente-deux des 38 patients positifs avaient été détectés par culture d'expectoration. La microscopie en DFA est capable de donner des renseignements diagnostiques rapides, mais elle ne peut être utilisée pour éliminer les diagnostics. L'expectoration est un prélèvement utile pour le premier examen de laboratoire des patients atteints de légionellose.

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LEGIONELLA PNEUMOPHILA IS A SIGNIFICANT CAUSE OF NOSOCOMIAL pneumonia. Early recognition and treatment is important, and therefore rapid means of diagnosis are desirable. Culture is the most sensitive technique, but results may not be available for up to nine days (1). Serology is of use as a retrospective means of diagnosis. Antigen detection is possible using latex agglutination (2), radioimmunoassay (3), enzyme-linked immunosorbent assay (4), nucleic acid probe (5) and immunofluorescence microscopy (6). A direct immunofluorescent staining method (DFA) has been used for many years in our laboratory, in combination with culture. This paper reviews our experience with culture and immunofluorescent staining of legionella using two sets of criteria to define a positive result for the DFA.

METHODS

In the first year of the study (January to December 1987) 515 specimens were submitted for legionella DFA and culture from 353 patients. In the second year (1988) there were 538 specimens from 385 patients (Table 1). Specimens were cultured on buffered charcoal yeast extract agar with alpha-ketoglutarate (BCYE), and on two selective media: BCYE supplemented with cefamandole (4 mg/L), polymyxin B (80,000 u/L) and anisomycin (80 mg/L); and BCYE with polymyxin (40,000 u/L), anisomycin (80 mg/L) and vancomycin (0.5 mg/L) (Gibco, Wisconsin) (7). In addition, a sheep blood agar plate was used to assess other flora as routine sputum cultures are performed elsewhere in the laboratory. Fluid specimens were concentrated by centrifugation, and tissues were homogenized and diluted 1:100 and 1:10,000 in sterile water prior to culture. The cultures were incubated at 37°C in 5% carbon dioxide and examined daily using a stereomicroscope for up to one week. Isolates were identified using direct immunofluorescence and the inability to grow on blood agar.

DFA was performed using a polyclonal fluorescein isothiocyanate (FITC)-labelled rabbit immunoglobulin directed against *L pneumophila* serogroup 1, Knoxville strain (Centers for Disease Control, Georgia). Slides were stained in accordance with the manufacturer's instructions. Briefly, slides were prepared in a safety cabinet, air dried, heat and formalin fixed, and stained with the antisera using laboratory prepared reagents. A negative control with FITC-labelled nonspecific rabbit antisera was included with each set of slides to be stained. A positive slide had at least one brightly fluorescing apple green rod seen in the test slide (8), with no fluorescing rods seen in the negative control. DFA was performed without knowledge of the culture result of the specimen.

Turn around times for culture were measured retrospectively by review of laboratory records, and defined as the number of days from the day of receipt in the laboratory until the day the final report was

TABLE 1
Number and types of specimens received for direct immunofluorescent assay and culture of legionella

Specimen type	1987 (n=353)	1988 (n=385)
Sputum	383	408
Bronchial wash	94	108
Tissue	30	18
Sterile fluid	7	4
Post mortem lung swab	1	0
Total	515	538

n Number of patients

issued. All positive reports were telephoned to the patient's unit. DFA was performed on the day of receipt or on the next working day if the specimen was received outside normal working hours.

In the second year, methods for culture remained the same. DFA was performed using reagents prepared with commercial steam sterilized water (sterile water for irrigation USP, Baxter Corp), as previous cultures of faucets in the laboratory had been positive for *L pneumophila*. A positive result was defined as having at least five typically staining organisms per slide (9).

Staining procedures with DFA reagents were reviewed to ensure positive slides were separated from others. The reagents, including the formalin, the swabs used and sterile water were culture-negative for legionella. DFA to detect viable but nonculturable organisms was also negative. Reagent container design was changed to minimize the danger of airborne contamination of reagents.

Specimen results were analyzed as either sputa or deep specimens, which included bronchial washes, tissues, pleural fluids and a post mortem lung swab. Culture was used as the reference technique. Specimens giving positive DFA results with negative cultures were interpreted as being false positive. The DFA results were analyzed to calculate sensitivity, specificity, positive and negative predictive values, and efficiency relative to culture. The results for each year were statistically analyzed using Fisher's exact test for low numbers or the χ^2 test.

RESULTS

During the first year there were 27 culture-positive specimens from 23 patients. In the second year there were 19 culture-positive specimens from 16 patients. The number of discrepant results is shown in Table 2.

Over the two years of the study, 38 patients were identified with infection with *L pneumophila* serogroup 1. A further patient's cultures yielded *Legionella bozemanii*; the DFA was negative as expected. Thirty-two of these 38 positive patients (84%) had positive sputum cultures. These cases occurred sporadically throughout the period, and no point source of infection was implicated.

The mean of the turn around times for sputum culture was five days (95% confidence interval, 4.45 to 5.54) and for bronchial wash specimens was 5.4 days (95% confidence interval, 3.93 to 6.92).

The analysis of the performance of the DFA relative to culture is summarized in Table 3. There was no statistically significant difference in the sensitivity, specificity, predictive values or efficiency between the two years.

DISCUSSION

The legionella DFA has been shown previously to be a specific rapid test for the diagnosis of legionellosis (10). Our results confirm this finding. The DFA was specific for both sputum and deep specimens.

False positive results may arise either because of other species of microorganisms cross-reacting with the antisera used, or from environmental *L pneumophila* contaminating the specimen. The similar specificities for sputum and deep specimens, despite the oropharyngeal flora, suggest that cross-reaction by other species was not the major cause of false positives. Culture for anaerobes and exhaustive screening for cross-reacting organisms, however, were not performed. Contamination of specimens with legionella may occur either from the patient, the container or in the laboratory. The patient may be colonized (8,11) may have been treated successfully, or may be transiently contaminated as a result of drinking or mouth rinsing with colonized potable water. The specimen may be contaminated by airborne organisms or splashes from contaminated potable water; the container may have been previously contaminated during production and irradiated, resulting in nonviable but morphologically intact organisms. Many of the modes of contamination outside the laboratory would introduce viable organisms to the specimen. As culture is much more sensitive than DFA, cultures could be expected to be positive if these modes occurred. The dilution of nonviable organisms introduced to a specimen would require a large inoculum if DFA were to be rendered positive as a result. In the laboratory, contamination may occur via the airborne route or by contamination of reagents which, when used in the preparation of stains, could allow large numbers of viable or nonviable organisms to be introduced. Phosphate buffered saline has been determined to be the source of false positives in one study; the incidence of false positives dropped following replacement of the reagents (12). In the present study, replacement of reagents did not result in a statistically significant change in the number of false positive results.

The sensitivity of DFA is not sufficient for it to be used to 'rule out' infection with legionella. However, provided clinicians recognize this limitation, it can provide useful data rapidly. Sixteen of 37 sputa (43%) and six of nine deep specimens (67%) were detected using

TABLE 2

Results of culture and direct immunofluorescent assay (DFA) for sputum and deep specimens in 1987 and 1988

Year	Culture	DFA	Sputum	Deep	Total
1987	+	+	11	4	15
	+	-	10	2	12
	-	+	14	5	19
	-	-	348	121	469
1988	+	+	5	2	7
	+	-	11	1	12
	-	+	2	0	2
	-	-	390	127	517

TABLE 3

Summary of the performance of direct immunofluorescent assay relative to culture in 1987 and 1988

	Overall		Sputa		Deep	
	1987	1988	1987	1988	1987	1988
Sensitivity	55.5	37	52.4	31.3	66.6	66.6
Specificity	96.1	99.6	96.1	99.5	96	100
PPV	44.1	77.8	44	71.4	44.4	100
NPV	97.5	97.7	97.2	97.3	98.4	99.2
Prevalence	5.2	3.5	5.5	3.9	4.5	2.3

NPV Predictive value of a negative test; PPV Predictive value of a positive test

the DFA. Clinicians must consider the results in light of the pretest probability of disease because of the low positive predictive values, and culture should always be used to confirm DFA results.

Sputum was a useful specimen. Thirty-two of 38 patients with legionellosis were detected using sputum culture. As the DFA can provide information within hours of a specimen being taken, initial examination of sputum samples may avoid the need for more invasive procedures in many patients, and specific treatment may be initiated earlier in the illness. Clinicians should not assume cultures are negative for at least six days after specimens are set up. Where legionella is suspected as a possible cause of pneumonia, treatment should include coverage for legionella for the full course or until it is no longer suspected. The DFA result is useful to supplement clinical impressions to influence decisions made concerning antimicrobial therapy.

The prevalence of positive specimens in the study was high possibly because of an increased incidence relative to other institutions, and careful selection of patients that had cultures taken. Legionella cultures were not performed as part of the routine sputum culture. The prevalence of disease influences the predictive values of a test. In centres with low prevalence, below 0.5% of specimens examined, the positive predictive value may fall to unacceptable levels despite the specificity being 99.5%. Strict control of the specimens examined may increase the prevalence in the specimens examined, but maintenance of expertise of reading the DFA may be difficult.

The number of fluorescing organisms required to call a specimen positive was changed from at least one to at least five. Unfortunately, because of the test's clinical importance, this change was made when reagents were altered to minimize possible contamination. The second year results, however, did not show a statistically significant change in test specificity and although the sensitivity dropped from 52.4 to 31.3% for sputum specimens (11 of 21 in 1987 versus five of 16 in 1988, two tailed $P=0.54$), this change did not reach statistical significance. Use of the less stringent criterion for positivity did not result in a significant loss of specificity and therefore should be preferred for routine use.

Sputum is the initial specimen of choice for the laboratory diagnosis of legionellosis because it is easy to obtain and noninvasive. Many patients may be diagnosed rapidly using the DFA, and the majority detected by culture. The DFA of sputum using the criteria of more than one organism per slide for a positive result was a useful procedure where the prevalence of infection is sufficiently high to yield satisfactory predictive values and maintain expertise. The sensitivity of the DFA from either sputa or deep specimens is insufficient to rely on DFA to exclude the diagnosis of legionellosis.

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