Technical methods

Serogrouping of *Legionella pneumophila* by double diffusion and counter immunoelectrophoresis

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Ten serogroups of *Legionella pneumophila* have now been described.1 Although the direct fluorescent antibody technique is widely used to identify and serologically differentiate *L. pneumophila* isolates; some cross reactivity between serogroups3 and some staining of non-legionella organisms with legionella antisera4 have been reportea. Several simple alternatives to the direct fluorescent antibody technique have been described which do not require fluorescence microscopes and competent microscopists. These include slide agglutination,2 staphylococcal coagglutination,3 latex agglutination,4 and antiserum-agar plates.7

Counter immunoelectrophoresis and double diffusion have been used to detect human antibodies to *L. pneumophila.*8,9 The studies reported here suggest that both double diffusion and counter immunoelectrophoresis can be used as alternatives to direct fluorescent antibody when serogrouping isolates of *L. pneumophila.*

Material and methods

Antisera to *L. pneumophila* serogroups 1 to 6 were obtained from the Division of Microbiological

Accepted for publication 30 May 1984

(a) Counter immunoelectrophoresis slide (unstained) and (b) double diffusion slide (stained) showing urea extracts of *L. pneumophila* strains tested against DMRQC serogroup 1 antiserum.

Counter immunoelectrophoresis wells: (A) antiserum, (1) serogroup 6 strain (Oxford-1), (2) serogroup 1 strain (Philadelphia-1), (3) serogroup 1 strain (Pontiac-1).

Double diffusion wells: (A) antiserum, (1) serogroup 1 strain (Philadelphia-1), (2) serogroup 1 strain (East Birmingham EB-1), (3) serogroup 2 strain (EB-2), (4) serogroup 3 strain (EB-3), (5) serogroup 4 strain (EB-4), (6) serogroup 5 strain (Cambridge-2), (7) serogroup 1 strain (Pontiac-1), (8) serogroup 6 strain (Oxford-1).
Results of testing 35 *Legionella pneumophila* strains, 132 non-**Legionella** strains, and calorifier and lung samples containing *L. pneumophila* by counter immunoelectrophoresis and double diffusion

<table>
<thead>
<tr>
<th><em>L. pneumophila</em> antisera</th>
<th><em>L. pneumophila</em> strains</th>
<th>Other bacterial species</th>
<th>Calorifier* deposit</th>
<th>Lung samples†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serogroup 1</td>
<td>Serogroup 1</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Serogroup 2</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Serogroup 3</td>
<td>-</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Serogroup 4</td>
<td>-</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Serogroup 5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Serogroup 6</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Pooled 1–6</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

* L. pneumophila* serogroup 1 isolated.
† All from positive cases of Legionnaires' disease.
NT = not tested.
+ = positive reaction.
− = negative reaction.

Reagents and Quality Control (DMRQC), Colindale, London. Fluorescent antibody titres were between 4000 and 16 000.

An antiserum to *L. pneumophila* serogroup 1 was also prepared in rabbits immunised with an ultrasonically disrupted antigen.* This was designated Phil-1 antiserum. Fluorescent antibody titre was 5000.

Antigens for counter immunoelectrophoresis and double diffusion were extracted from 24 or 48 h old cultures on buffered charcoal yeast extract agar (Oxoid Ltd, Basingstoke, England). Organisms under test were suspended in 35 µl of phosphate buffered saline (PBS) in a glass bijou bottle to about 3 × 10⁹ colony forming units (CFU) /ml (roughly equivalent to two 3 cm sweeps through confluent growth). Diethyl ether (100 µl) was added, and the contents were shaken. The bottle was incubated at room temperature for 10 min and then 200 µl of 9 M urea was added, mixed, and the bottle left for a further 10 min. The mixture was centrifuged for 5 min at 2000 g and the supernatant was used as antigen.

Counter immunoelectrophoresis was performed as described previously* except that the antigen and antibody wells were 3 mm in diameter and 3 mm apart and 10 µl volumes were employed in each (Fig (a)).

Double diffusion was carried out on glass microscope slides with 3 ml of 1% agarose (BDH) in PBS, pH 7·2. Nine wells (3 mm diameter) were made: one central antiserum well and eight others at 3 mm from it for antigen extracts (Figure (b)). The slides were incubated at room temperature overnight in a moist chamber.

Results

Serogroups determined by counter immunoelectrophoresis and double diffusion showed 100% correlation with previously determined fluorescent antibody serogroups when 35 strains of *L. pneumophila* were tested (Table). No cross reactions between serogroups were seen. No reactions were noted when these 35 strains were tested against pooled serogroup 1 to 6 antiserum, or when 132 non-legionella strains were tested against serogroup 1 antiserum. A calorifier deposit known to contain *L. pneumophila* and four lung samples from confirmed cases of Legionnaires' disease were repeatedly negative with all antiserum by both counter immunoelectrophoresis and double diffusion.

Discussion

A positive counter immunoelectrophoresis or double diffusion result was denoted by the appearance of a single slightly diffuse, almost central, precipitin line (Figure), although one serogroup 3 strain and four serogroup 1 strains occasionally produced negative results when tested. These strains were all positive when repeated. No morphological differences were seen between these strains and others apart from slightly less luxuriant growth and a tendency for the urea extract to be slightly sticky after centrifugation; however, these occasional false negative results mean that all organisms examined by these techniques should be extracted and tested in duplicate in an attempt to eliminate this problem. Strains which persist in giving a negative reaction should be examined by conventional means or sent to a reference laboratory. *L. pneumophila* strains cultured on legionella blood agar (Oxoid) gave comparable results to strains grown on charcoal yeast extract agar when tested by counter immunoelectrophoresis and double diffusion. Occasional non-legionella isolates such as *Pseudomonas*
fluorescens and Bacteroides fragilis have been shown
to give positive reactions with L pneumophila anti-
sera by double diffusion and direct fluorescent anti-
body,4 owing to shared antigens, but none of the 132
non-legionella strains tested here (which included
13 P fluorescens and 33 B fragilis) produced any
reaction with serogroup 1 antisera (DMRQC or
Phil-1) by counter immunoelectrophoresis or dou-
ble diffusion.

The results indicate that counter immunoelec-
trophoresis and double diffusion are not sufficiently
sensitive to detect the relatively small amount of
antigen presumably present in clinical or environ-
mental samples such as calorifier deposits and lung
tissues. The lack of sensitivity of counter immuno-
electrophoresis in detecting legionella antigens
has been noted previously.10

Both counter immunoelectrophoresis and double
diffusion can be used to group and identify strains of
L pneumophila accurately. Double diffusion is a
much simpler and more economic test than counter
immunoelectrophoresis when testing multiple
strains, as up to eight extracts can be tested simul-
taneously against 10 μl of each antiserum, whereas
each extract tested requires 10 μl of antiserum by
counter immunoelectrophoresis. Thus although
both techniques could be used in laboratories where
fluorescent antibody techniques are not available,
double diffusion should be the method of choice.

The fact that none of the L pneumophila strains
reacted with the pooled serogroup 1–6 antiserum is
probably due to the consequent dilution of each
individual antiserum, and it is suggested that only
next, high titre antiserum such as those obtained from
DMRQC should be used for counter immunoelec-
trophoresis and double diffusion.

We thank Dr AG Taylor, Division of Microbial
Reagents and Quality Control, Colindale, London,
for kindly supplying antiserum and strains of L
pneumophila, and for much helpful advice and
encouragement; Dr AD Macrae, Queen’s Medical
Centre, Nottingham, for kindly supplying positive
lung tissue; and Dr J Gray and Mr A Barratt, PHLS,
Stoke on Trent, for kindly supplying Phil-1 anti-
serum. Mrs D Johnson kindly typed the manuscript.

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New medium for enhancing pig-
ment production of group B streptococci

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Group B streptococci are important pathogens in
neonates12 and in adults.14 It is important to identify
group B streptococci either by serogrouping or by
demonstrating one of their unique physiological
characteristics. The sensitive and specific physiologi-
ical method is pigment production.15 Specific
media16 for enhancing pigment production of
group B streptococci need anaerobic conditions and
contain serum. The purpose of this study was to
develop a new medium which does not require
serum and anaerobic conditions.
Serogrouping of Legionella pneumophila by double diffusion and counter immunoelectrophoresis.
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*J Clin Pathol* 1984 37: 1308-1310
doi: 10.1136/jcp.37.11.1308

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