A rapid method for staining inclusions of Chlamydia psittaci and Chlamydia trachomatis

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SUMMARY A new staining method was developed for the detection of inclusions of Chlamydia psittaci and Chlamydia trachomatis inclusions in cell cultures. Using a combination of methyl green and neutral red stains and washing at pH 5.0, inclusions were stained red while cell cytoplasm was pale pink and cell nuclei were pale green. The method was significantly better than Giemsa staining and comparable to immunofluorescence for detecting C. psittaci inclusions. Its sensitivity for detecting C. trachomatis inclusions by dark field microscopy was similar to that of Giemsa staining.

A variety of staining methods have been used to visualise chlamydial inclusions in cell culture monolayers and conjunctival scrapings.1-2 Giemsa stain, combined with dark field illumination, has been commonly used to detect Chlamydia trachomatis inclusions in cell culture monolayers, but this has not proved to be satisfactory for detecting Chlamydia psittaci inclusions.3-4 While such inclusions can be seen by bright field microscopy, the contrast is poor and they can be difficult to detect by low power microscopy. Iodine stain5 and carmine stain6 detect the glycogen matrix of inclusions and are therefore only of use for mature C. trachomatis inclusions. Immunochromic staining methods, such as immunofluorescence (FA) and immunoperoxidase, are sensitive and reliable but require the use of expensive reagents. A methylene blue staining method for detecting ewe abortion agent (C. psittaci) inclusions has been described,3 but its efficacy for other chlamydiae is not known.

In our laboratory, studies of human C. psittaci infections and of animal diseases caused by the agents of feline keratoconjunctivitis (FKC) and guinea-pig inclusion conjunctivitis (GPIC), which are both members of C. psittaci, as models for the study of human chlamydial infections, led to the need for a reliable, practical and inexpensive method by which inclusions produced by these organisms could be detected in cell culture monolayers. A new simple staining method, which uses methyl green and neutral red (MG-NR), has been developed for staining inclusions of both species of chlamydiae. Its sensitivity was compared with the sensitivities of the standard method of Giemsa staining, the methylene blue method, and immunofluorescence staining (FA) for detecting C. psittaci and C. trachomatis inclusions.

Material and methods

CHLAMYDIAL ISOLATES The following C. psittaci agents were used to infect McCoy cell monolayers: feline keratoconjunctivitis agent (FKC) strain FKC/Ps/1/IOL-457/O; guinea pig inclusion conjunctivitis agent (GPIC) strain A10; meningoencephalitis agent (MPV) strain Cal-10; ewe abortion agent (EA); a human genital C. psittaci isolate—strain 33L; a human ocular C. psittaci isolate—strain IOL-395; and an atypical iodine-negative chlamydial agent—strain IOL-207.7 In addition, an ocular genital C. trachomatis agent, strain TRIC/D/USA-Cal/Cal-15/ON, was used.

CLINICAL SPECIMENS Conjunctival and urethral swabblings were collected from guinea pigs which had been inoculated with GPIC agent or FKC agent and from rabbits which had been inoculated with FKC agent as part of another investigation. These swabblings were stored at -70°C until inoculated on to McCoy cell monolayers.

CELL CULTURE INOCULATION Laboratory isolates and animal specimens were cultured in cycloheximide-treated McCoy cells. The inoculated monolayers were incubated for 48 h in the case of FKC agent and 72 h for the other chlamydiae. They were then fixed in absolute methyl alcohol for 10 min and stained using either methyl
Rapid staining of chlamydiae

green-neutral red (MG-NR), Giemsa, 0-5 % methyl-
ene blue solution, or immunofluorescence (FA).

THE MG-NR STAINING METHOD
Stock solutions of methyl green and neutral red were
preared as follows:
An aqueous solution (1 % wt/vol) of methyl green
(Raymond A Lamb, 6 Sunbeam Road, London
NW10) was prepared and extracted several times with
chloroform until all traces of blue dye were removed.
A neutral red solution (0-1 % wt/vol) was prepared by
dissolving one gram of neutral red (GT Gurr, BDH,
Poole, Dorset) and 2 ml of 1% acetic acid in one
litre of distilled water. The resulting solution was
filtered immediately. These stock solutions have
been stored at room temperature for several months
with no apparent deterioration.
For use, a mixture consisting of nine parts methyl
green solution and one part neutral red solution
was prepared. This was usually used immediately but
satisfactory staining has been achieved using mixtures
which had been prepared and stored for at least two
weeks.
Fixed cell monolayers were washed with a pH
5-0 buffer solution and then stained for 10 min in the
methyl green-neutral red mixture. The mono-
layers were then washed in pH 5-0 buffer solution
for 10 min, air-dried, and mounted in Gurr’s
‘Uvinert’ mountant (BHD, Poole, Dorset). Cell
nuclei stained pale blue-green, cytoplasm was very
pale pink, and inclusions were stained red.

EXAMINATION OF STAINED MONOLAYERS
Giemsa, methylene blue and MG-NR stained
monolayers were examined by normal bright field
microscopy or by dark field illumination. Immu-
ofluorescence-stained monolayers were examined
using a Zeiss fluorescence microscope fitted with an
epi-illumination system.

DESIGN OF EXPERIMENTS
To compare the Giemsa, methylene blue and MG-
NR staining methods, each laboratory isolate was
inoculated on to six monolayers, two of which were
stained by each method. The average number of
inclusions seen per monolayer was calculated from
the numbers counted in at least 30 microscope
fields (x 500 magnification) on each of the duplicates.
To mimic clinical specimens containing few infec-
tive particles, a number of dilutions of FK agent
were each inoculated on to 10 McCoy cell
monolayers, five of these were stained with MG-NR
and five with Giemsa. Each monolayer was examined
(at x 200 magnification) by two observers on a
double blind basis.
Clinical specimens were each inoculated on to two
monolayers, one of which was stained with MG-NR
and the other using either Giemsa or FA. The
monolayers were coded and examined (at x 200
magnification) for the presence of inclusions by
two observers on a double blind basis.

Results
No significant difference was observed between the
numbers of inclusions counted (x 500 magnification)
using the MG-NR, methylene blue and Giemsa
methods for the eight different chlamydial strains
tested (Table 1) (p > 0.05) but when specimens
containing few infectious C psittaci particles were
used and monolayers were examined by low power
microscopy (x 200 magnification), MG-NR was
superior to Giemsa staining (Table 2).

Table 1 Average numbers of inclusions detected in
McCoy cell monolayers inoculated with chlamydial
isolates after staining with Giemsa, methylene blue or
methylene green-neutral red

<table>
<thead>
<tr>
<th>Chlamydia</th>
<th>Giemsa</th>
<th>Methylene blue</th>
<th>Methylene green-neutral red</th>
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</thead>
<tbody>
<tr>
<td>FKC</td>
<td>1.1 x 10^4</td>
<td>4.2 x 10^4</td>
<td>6.6 x 10^4</td>
</tr>
<tr>
<td>GPIC</td>
<td>2.5 x 10^4</td>
<td>2.0 x 10^4</td>
<td>2.3 x 10^4</td>
</tr>
<tr>
<td>IOL 207</td>
<td>1.4 x 10^4</td>
<td>1.5 x 10^4</td>
<td>1.5 x 10^4</td>
</tr>
<tr>
<td>33L</td>
<td>4.7 x 10^4</td>
<td>4.8 x 10^4</td>
<td>7.5 x 10^4</td>
</tr>
<tr>
<td>EA</td>
<td>1.8 x 10^4</td>
<td>1.8 x 10^4</td>
<td>1.8 x 10^4</td>
</tr>
<tr>
<td>MPV</td>
<td>5.0 x 10^4</td>
<td>5.3 x 10^4</td>
<td>5.7 x 10^4</td>
</tr>
<tr>
<td>395</td>
<td>1.0 x 10^4</td>
<td>1.3 x 10^4</td>
<td>1.7 x 10^4</td>
</tr>
<tr>
<td>Cal-15</td>
<td>2.2 x 10^4</td>
<td>1.6 x 10^4</td>
<td>1.3 x 10^4</td>
</tr>
<tr>
<td>Cal-15*</td>
<td>2.2 x 10^4</td>
<td>2.6 x 10^4</td>
<td>2.4 x 10^4</td>
</tr>
</tbody>
</table>

*Inclusions counted by dark field illumination.

Table 2 Comparison of methyl green-neutral red
and Giemsa staining for the detection of FK agent
inclusions in specimens with low infectivity titres

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Methyl green-neutral red</th>
<th>Giemsa</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Observer 1</td>
<td>Observer 2</td>
</tr>
<tr>
<td>10^-4</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10^-3</td>
<td>3/5</td>
<td>4/5</td>
</tr>
<tr>
<td>10^-4</td>
<td>2/5</td>
<td>1/5</td>
</tr>
<tr>
<td>10^-1</td>
<td>0/5</td>
<td>1/5</td>
</tr>
</tbody>
</table>

For the C psittaci strains, the MG-NR method
produced clearly stained inclusions which were well
differentiated and were easily detected by bright
field illumination in every case. In our hands,
the differentiation achieved with methylene blue
appeared in general to offer little advantage over
Giemsa. In the case of the C trachomatis strain,
MG-NR stained inclusions (pale pink) were poorly
differentiated from the background when viewed by
bright field illumination, but could be distinguished easily when dark field illumination was used.

Of 96 conjunctival swabblings taken from guinea pigs and rabbits experimentally infected with FKC agent, 66 (69%) were positive by MG-NR, but only 56 (58%) by Giemsa. Three specimens were only positive by Giemsa and 13 specimens were positive by MG-NR alone. The difference between these two methods was statistically significant (p < 0.05).

A separate series of 66 conjunctival and urethral swabblings taken from guinea pigs inoculated experimentally with GPIC agent was used to compare the MG-NR staining method with FA staining; 35 (53%) were positive by MG-NR and 37 (56%) by FA. Five specimens were positive by FA alone while three were positive only by MG-NR. The difference between the results for these two methods was not significant (p > 0.05).

Discussion

*Chlamydia psittaci* infections occur commonly in animals and are often transmitted to humans to cause disease. Isolation in tissue culture has not been widely used for the diagnosis of these diseases, possibly because the inclusion bodies formed in cell culture monolayers by many of these organisms cannot be reliably detected by the Giemsa or iodine staining methods which have been used routinely for *C. trachomatis*.3 4

The results of this study show that the MG-NR staining method is more sensitive than Giemsa and is comparable in sensitivity to FA staining for detecting *C. psittaci* inclusions. In addition, *C. trachomatis* inclusions could be readily detected by dark field microscopy, thereby allowing the staining method to be used for detecting both *C. psittaci* and *C. trachomatis* inclusions. It is quick and easy to do, producing slides ready for examination in about 30 min, which is shorter than the time needed for either Giemsa or indirect immunofluorescence. The greater contrast achieved by the MG-NR method produced an additional saving in time that monolayers could be examined rapidly at low magnification. It has proved to be suitable for routine use for the detection of both species of chlamydiae; examination being carried out by bright field or dark field illumination, or both, as required.

As part of the MG-NR staining procedure, an acid (pH 5-0) differentiation stage was used to increase the contrast between the inclusions and the cells. Acid conditions have been used at some stage in many of the staining procedures which have been described for chlamydiae but the importance of this does not appear to have been emphasised. In addition to MG-NR, we have noticed that many other dyes, such as Giemsa, May-Grünwald stain, malachite green, methylene blue, and toluidine blue, gave a much better contrast between inclusions and cells when used at pH 5-0 than at the standard pH 6-8 to 7-2 which is recommended for Giemsa by the World Health Organisation.10

The authors wish to thank Mr R Hejazi and Mrs M Pink for assistance with clinical specimens. This work was supported by grants from the Department of Health and Social Security, an anonymous donor, and the Locally Organised Research Scheme, Moorfields Eye Hospital.

References

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*J Clin Pathol* 1982 35: 642-644
doi: 10.1136/jcp.35.6.642

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