

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. Immunochemical staining methods, such as immunofluorescence (FA) and immunoperoxidase,⁴ 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; meningopneumonitis 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 oculogenital *C trachomatis* agent, strain TRIC/D/USA-Cal/Cal-15/ON, was used.

CLINICAL SPECIMENS

Conjunctival and urethral swabbings 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 swabbings 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.⁸ 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

green-neutral red (MG-NR), Giemsa, 0.5% methylene blue solution,³ or immunofluorescence (FA).⁴

THE MG-NR STAINING METHOD

Stock solutions of methyl green and neutral red were prepared 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 monolayers 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. Immunofluorescence-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 ($\times 500$ magnification) on each of the duplicates. To mimic clinical specimens containing few infective 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 $\times 200$ 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 $\times 200$ 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 ($\times 500$ 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 ($\times 200$ 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 methyl green-neutral red

Chlamydia	Giemsa	Methylene blue	Methyl green-neutral red
FKC	1.1×10^3	4.2×10^3	6.6×10^3
GPIC	2.5×10^3	2.0×10^3	2.3×10^3
IOL 207	1.4×10^4	1.5×10^4	1.5×10^4
33L	4.7×10^3	4.8×10^3	7.5×10^3
EA	1.8×10^3	1.8×10^3	1.8×10^3
MPV	5.0×10^3	5.3×10^3	5.7×10^3
395	1.0×10^3	1.3×10^3	1.7×10^3
Cal-15	2.2×10^3	1.6×10^3	1.3×10^3
Cal-15*	2.2×10^3	2.6×10^3	2.4×10^3

*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

Dilution	No of positive monolayers			
	Methyl green-neutral red		Giemsa	
	Observer 1	Observer 2	Observer 1	Observer 2
10^{-4}	5/5	5/5	0/5	0/5
10^{-5}	3/5	4/5	0/5	0/5
10^{-6}	2/5	1/5	0/5	0/5
10^{-7}	0/5	1/5	0/5	0/5

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 swabbings taken from guinea pigs and rabbits experimentally infected with FK 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 swabbings 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 in 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^{3 6 9} 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

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