Number of Bordetella pertussis strains isolated on Bordet-Gengou medium or charcoal agar medium, or both, during 1980–1982

Year	Total no of specimens	Total no of positive cultures	Growth on Bordet-Gengou medium only	Growth on charcoal agar medium only	Growth on both Bordet-Gengou and charcoal agar medium
1980	275	59	6 (10·6%)*	14 (23·7%)	39 (66·1%)
1981	233	34	4 (11·7%)	11 (32·3%)	19 (55·8%)
1982	411	80	12 (15%)	20 (25%)	48 (60%)

*Figures in parenthesis present the percentage of the total positive culture.

media is justified. A good isolation rate of *B pertussis* depends on several factors and we consider that one important factor is the use of two media in parallel.

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Microimmunofluorescence technique for detection of antibody to *Mycoplasma genitalium*

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Mycoplasma genitalium organisms were isolated originally from the urethras of men suffering from non-gonococcal urethritis.[1,2] They have proved to be fastidious in their growth requirements, slow to replicate, and difficult to isolate.[3] The metabolism inhibition test[4] may be used as a serological approach to determine the possible association of this mycoplasma with disease, but slow multiplication of the organisms delays a result and antibiotics in serum may inhibit mycoplasmal growth and lead to a false result. We have developed a microimmunofluorescence technique for detecting antibodies to *M genitalium* which does not have these drawbacks and appears to be more sensitive than the metabolism inhibition test. Furthermore, this mycoplasma, like *M pneumoniae,* has the ability to adhere to glass or plastic, a property which facilitates the production of antigen.

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Material and methods

ANTIGEN PREPARATION

M genitalium was used after four subcultures in SP4 liquid medium. One millilitre of a culture containing 5×10^6 colour changing units was added to 40 ml of SP4 medium in a 260 ml capacity plastic tissue culture flask (Nunc). The flask was incubated horizontally at 37°C until the colour of the medium had changed from red to yellow, a period of about eight days. By this time a sheet of mycoplasmas was

adherent to the plastic. The growth medium was discarded and the sheet was washed with 20 ml of sterile phosphate buffered saline (PBS), pH 7·2, after which a few glass beads were agitated gently to remove the mycoplasmas from the plastic surface. The resulting suspension was centrifuged at 4000 rpm for 30 min in a Super Minor bench centrifuge (MSE), and the supernatant fluid was removed. The deposit was washed in PBS, resuspended with the aid of a Rotamixer (Hook and Tucker Instruments), and centrifuged as before. This procedure was undertaken three times, and the final deposit was resuspended in 1·0 ml of PBS and stored in 0·1 ml aliquots at −20°C. The protein concentration of the antigen was determined[5] and it was diluted in PBS to 0·75 mg protein/ml for use.

FLUORESCEIN LABELLED CONJUGATE

A freeze dried batch of fluorescein conjugated sheep antihuman globulin (Wellcome) was reconstituted in distilled water and stored in 0·1 ml amounts at −20°C. An aliquot was thawed at the time of the test and diluted to the manufacturer's recommended working dilution. The dilution to be used for any subsequent batch was determined by testing it in parallel with the previous batch using the same antigen and same "positive" control serum (see below) as used previously.

MICROIMMUNOFLUORESCENCE TEST

The antigen was thawed, mixed thoroughly, and then applied with a mapping pen to clear areas on Teflon coated 3″ × 1″ microscope slides, as described by Thomas *et al.*[6] The antigen was allowed to dry on the slides at room temperature, after which they were transferred to acetone for 30 min to fix the antigen. Some of these slides were used immediately, while others were stored at −20°C.

Sera (25 μl volumes) were diluted in twofold steps up to a 1/256 dilution in U-shaped, polystyrene, microtitre plates using PBS, pH 7·2, as the diluent. The serial dilutions were transferred to the slides using a fine Pasteur pipette or an Eppendorf pipette delivering 15 μl, commencing at the highest dilution. Then the slides were placed in a moist chamber and incubated at 37°C for 30 min, after which they received three washes, each of 10 min, in PBS and were allowed to dry at room temperature. Fluorescein conjugated sheep antihuman globulin was applied to the slides, which were returned to the moist chamber for a further 30 min at 37°C before being washed as before and dried at room temperature. The slides were examined by epifluorescence microscopy (×100 and ×400 magnification) and green/yellow fluorescence was graded on an arbitrary + to +++ scale. The antibody titre was recorded as the highest dilution of serum at which a grade of + fluorescence was seen. A serum containing antibody of known titre ("positive" control) and a serum without detectable antibody ("negative" control) were included in each test. For comparison, antibody was also measured by the metabolism inhibition technique.[4]

Results

The results of testing sera from two female grivet monkeys and two marmosets inoculated genitally with *M genitalium* and also from two men with non-gonococcal urethritis are shown in the Table. They are representative of results obtained in tests on a larger number of similar sera. Usually the titre of antibody measured by the microimmunofluorescence test was greater than that measured by the metabolism inhibition test.

Antibody to M genitalium measured by microimmunofluorescence (MIF) and metabolism inhibition (MI) techniques

Source of serum	*Antibody titre (reciprocal) measured by*	
	MIF	*MI*
Grivet monkeys		
postinoculation (after 4 wk)	128	16
,, ,, ,,	64	16
Marmosets		
preinoculation	<2	2
postinoculation (after 3 mo)	64	8
preinoculation	2	2
postinoculation (after 3 mo)	32	8
Patients with non-gonococcal urethritis		
Acute phase	16	8
Convalescent phase (4 wk later)	4	4
Acute phase	32	2
Convalescent phase (2 wk later)	64	2

Several aspects of the microimmunofluorescence test indicated that it was reproducible. Firstly, the antibody titre of a "positive" control serum did not vary in six consecutive tests with the same antigen and conjugate. Secondly, the antibody titres of eight human and marmoset sera when retested were no more than twofold different from the titres obtained originally. Thirdly, the results of tests undertaken on slides which had been stored at −20°C for up to three months were similar to those of tests carried out on freshly prepared slides.

Discussion

Other workers[7] have used mycoplasma colonies on agar as the source of antigen in immunofluorescence tests mainly to identify mycoplasmas. With that method colonies are produced freshly for each test whereas the antigen in the method we describe may be prepared in advance and stored frozen in small aliquots until required. This means also that the antigen is the same in each test and not subject to variation owing to changes in the components of the agar medium and age of the culture. In addition, slides spotted with antigen may be prepared in advance and stored until required.

The antigen for the microimmunofluorescence technique described here was prepared by allowing *M genitalium* organisms to attach to and grow as a sheet on the surface of a plastic tissue culture flask. This facilitated washing of the antigen. It was important to resuspend the organisms evenly after storage and before application to the slides as there was a tendency for them to clump. Acetone fixation of the antigen was used routinely for convenience, but heat fixation produced results which were comparable. Use of the microimmunofluorescence test overcomes the problems which may be encountered when using viable organisms, as in the metabolism inhibition test, where antibiotics in the serum may inhibit multiplication and thereby give rise to spurious results. We used the microimmunofluorescence test successfully to detect antibody to *M genitalium* in sera from men with non-gonococcal urethritis and in sera from grivet monkeys and marmosets inoculated with this mycoplasma.[8] It was possible to use the fluorescein labelled antihuman globulin for the simian sera as there is cross reactivity between the human and simian species. It should be easy to adapt this test to differentiate between IgG and IgM antibodies, which is difficult to accomplish by the metabolism inhibition procedure. In most instances, the antibody titres measured in the microimmunofluorescence test were greater than in the metabolism inhibition test, indicating that the former procedure is more sensitive. Furthermore, the microimmunofluorescence technique requires very small volumes of all reagents, is quick to perform, easy to read, reproducible, and, of course, may be used for mycoplasmas other than *M genitalium*. In this regard, the results of preliminary microimmunofluorescence tests in which *M hominis* has been used as antigen show that antibody to this mycoplasma does not react with *M genitalium*. However, the latter is known to cross react with *M pneumoniae* in complement fixation[9] and, to a lesser extent, metabolism inhibition tests,[10] and the microimmunofluorescence technique offers another approach to defining this relation.

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