Characterisation of monoclonal antibodies for detection of *Mobiluncus* spp. in genital specimens

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Summary. Monoclonal antibodies were raised to the anaerobic curved rods, *Mobiluncus curtisii* subsp. *curtisii* NCTC 11656, *M. curtisii* subsp. *holmesii* NCTC 11657 and *M. mulieris* NCTC 11658. Three antibodies reacted with the two subspecies of *M. curtisii* and, when used in combination against clinical isolates, showed 100% sensitivity and specificity in immunofluorescence studies. Immunoblotting showed that two of these antibodies reacted with an epitope on a protein which had an electrophoretic mobility corresponding to a Mr of 75 Kda in the absence of a reducing agent and 82 Kda in its presence in both type strains and in clinical isolates. The third antibody reacted with an epitope in type strains which had a mobility corresponding to 75 Kda and was unaffected by a reducing agent. However, in clinical isolates the epitope was present on a protein of 75 Kda or 71 Kda, or on both. A fourth antibody showed reactivity with *M. mulieris* NCTC 11658 alone, but only 6 (24%) of 25 clinical isolates gave positive results by immunofluorescence. The epitope is believed to be present on a protein of >90 Kda. All four antibodies were shown by immunogold staining to be directed against epitopes exposed on the cell surface.

Introduction

Motile curved bacilli were first described in vaginal secretions by Curtis (1913) and then by Moore (1954) and Durieux and Dublanchet (1980). Spiegel and Roberts (1984) found that these bacilli were distinct from related genera and recommended the creation of a new genus *Mobiluncus* to include two species—*M. mulieris*, the long variant and *M. curtisii* the short variant, the latter being further divided into *M. curtisii* subsp. *curtisii* and subsp. *holmesii*. Characteristics of the genus include multiple polar flagella, a multilayered gram-variable cell wall, production of succinate and acetate, and a DNA G + C content of 49–52 mol%.

Anaerobic curved rods, together with *Gardnerella vaginalis* and *Bacteroides* spp., have been associated with bacterial vaginosis (Hjelm *et al.*, 1981; Phillips and Taylor, 1982; Skarin and Mardh, 1982; Sprott *et al.*, 1982). However, *Mobiluncus* spp. are fastidious, slow growing organisms and, in most studies, they have been isolated from only 10–50% of women with vaginosis (Sprott *et al.*, 1983; Hjelm *et al.*,

1984; Hallen *et al.*, 1987). This is in contrast with studies in which the diagnosis was based on microscopy; in these, motile curved rods have been seen in vaginal secretions from up to 90% of women with vaginosis (Roberts *et al.*, 1985). Using a DNA probe to detect *Mobiluncus* spp. in vaginal secretions, Roberts *et al.* (1985) found the organisms in 83% of women with vaginosis, suggesting that the low figures obtained in previous studies with culture resulted from technical problems. Unlike *G. vaginalis* and *Bacteroides* spp., curved motile rods have been reported only rarely in women without vaginosis (Skarin and Mardh, 1982; Spiegel *et al.*, 1983; Sprott *et al.*, 1983). Hallen *et al.* (1987) used monoclonal antibodies (MAbs) in an immunofluorescence test to detect *Mobiluncus* spp. in women with bacterial vaginosis. They provided evidence that immunofluorescence was more sensitive than microscopy of wet smears and also showed that *Mobiluncus* spp. could be detected in a few women who did not have signs of vaginosis. In this paper we describe the characterisation of four MAbs raised against *Mobiluncus* spp. for their potential use for the detection of *Mobiluncus* spp. in clinical specimens.

Materials and methods

Strains

The three type strains of *Mobiluncus* spp. were obtained from the National Collection of Type Cultures, Colindale, London—*M. curtisii* subsp. *curtisii* NCTC 11656, *M. curtisii* subsp. *holmesii* NCTC 11657, and *M. mulieris* NCTC 11658. Seventeen clinical isolates were from our own collection, 20 strains were obtained from Professor I. Phillips, St Thomas's Hospital, London and 25 from Dr M. S. Sprott, Public Health Laboratory, Newcastle-upon-Tyne. Of the 62 strains used, 37 were short curved rods and 25 were long curved rods.

Bacteria used to test the specificity of the antibodies in immunofluorescence studies included 9 strains of anaerobic curved rods which did not conform to the definition of *Mobiluncus* spp. but were not identified further, 10 strains of *Neisseria gonorrhoeae*, 20 of *G. vaginalis*, 10 of lactobacilli, 10 of Group B streptococci, 6 of *Haemophilus ducreyi*, one of *Peptostreptococcus*, 8 of *Bacteroides bivius*, 5 of *B. disiens*, 5 of *B. melaninogenicus* and one of *Escherichia coli*.

Isolation and identification

All strains of *Mobiluncus* spp. were grown on Columbia Agar Base (BBL Microbiology Systems) containing outdated banked human blood 10% (HBA); cultures were incubated for 3–4 days at 37°C in an anaerobic atmosphere generated by an Anaerocult A system without a catalyst (Merck Diagnostic, BDH).

Bacterial strains were identified as *Mobiluncus* spp. if they were anaerobic, motile, gram-variable curved rods which were resistant to a 5- μ g metronidazole disk. The two species were distinguished by the ability to hydrolyse hippurate, reduce nitrate and ferment carbohydrates by the use of methods described previously (Holdeman *et al.*, 1977). Enzyme production was detected with API-ZYM (API Laboratories) test strips. Strains were divided into the two species by the criteria described by Spiegel and Roberts (1984).

Production of MAbs

Male BALB/C mice were immunised with formalin-treated whole cells of *M. curtisii* subsp. *curtisii* NCTC 11656, *M. curtisii* subsp. *holmesii* NCTC 11657 or *M. mulieris* NCTC 11658. The mice were inoculated intramuscularly with 0.1 ml of a suspension containing 5.0×10^8 cells/ml in Freund's incomplete adjuvant and given a booster dose 9 weeks after the first injection. The spleen was removed for fusion 3 days after the last injection.

Spleen cells from the immunised mice were fused with uncloned NSO myeloma cells by the polyethylene glycol fusion method of Galfré and Milstein (1981). Hybridoma cell lines were selected in DMEM (Flow Laboratories) containing hypoxanthine-aminopterin-thymidine and fetal calf serum (Tissue Culture Services) 18% v/v. Hybrid-

oma supernate was screened for antibody production by an indirect ELISA in which whole cells of various strains of *Mobiluncus* spp., including the immunising strains, were used as antigen. Hybridoma cell lines were cloned at least twice by a limiting dilution method with BALB/C macrophage feeder layers, concentrated and stored at -192°C in fetal calf serum 90% and dimethyl sulphoxide 10%.

A 'capture' ELISA was used to determine the isotype of the MAbs. Sheep anti-mouse IgG2a (Serotec), and goat anti-mouse IgG1, IgG2b, IgG3 and IgM (Sigma) were diluted in 50 mM sodium carbonate buffer, pH 9.6, and coated on to PVC microtitration plates. After incubation with hybridoma culture supernate, goat anti-mouse IgG (specific for light and heavy chains) conjugated to alkaline phosphatase (Sigma) was added. The substrate used was *p*-nitrophenyl phosphate 1 mg/ml in 50 mM carbonate buffer, pH 9.8, containing 1 mM MgCl₂, and absorbance was read at 405 nm after 30 min.

Immunofluorescence microscopy

The antigen was prepared by growing *Mobiluncus* strains on 10% HBA as described above. A thin smear was made on a multispot slide in phosphate buffered saline (PBS), air dried and fixed in methanol 70%. Antibody was diluted in PBS and added to each spot, incubated at 37°C for 30 min and then washed in PBS followed by distilled water. Rabbit anti-mouse immunoglobulin conjugated to FITC (Dakopatts) was added, incubated as before, washed, dried and examined with a Laborlux K microscope (Leitz).

Immunoblotting of SDS-PAGE

Each *Mobiluncus* strain was grown on 10% HBA for 3–4 days, harvested, washed and suspended in saline. The protein concentration of the suspension was estimated (Lowry *et al.*, 1951) and adjusted to 2 mg/ml. The suspension was diluted in double strength sampling buffer (bromophenol blue 0.1%, sodium dodecyl sulphate 2%, 0.1 M Tris-HCl, pH 6.8, 8% v/v and glycerol 10%). Samples of the type strains and the clinical isolates were prepared in the presence and absence of 0.1 M Cleland's reagent (Sigma) and solubilised at 100°C for 10 min.

The cell lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) with either a 10% (short curved rods) or a 15% (long curved rods) separating gel and a 5% stacking gel on a Protean II apparatus (Bio-Rad). Samples containing 10 μ g of protein were loaded on to the gel with low-mol.-wt standards (Pharmacia) and the gel was run for 2 h at 30 mA/gel. Proteins were then transferred to nitrocellulose (Hybond C, Amersham International) electrophoretically in a buffer of 20 mM Tris, 150 mM glycine and methanol 20% in a Trans-Blot apparatus (Bio-Rad Laboratories) with a current of 200 mA for 2 h (Towbin *et al.*, 1979). The tracks containing mol.-wt markers were removed and

stained for protein with amido black 0.1% in methanol 25% and acetic acid 10%. The rest of the membrane was then immersed in bovine serum albumin (BSA) 4% in washing buffer (0.15 mM sodium chloride, 10 mM Tris, pH 7.4) and incubated at 37°C for 1 h. All further dilutions or washings were with the same buffer containing BSA 0.1%. Antibody was added at the appropriate dilution (1 in 1000 for MAb 180.1 and 1 in 500 for MAbs 179.1, 179.3 and 181.1) and left overnight at 4°C. After washing, the membranes were incubated with rabbit anti-mouse immunoglobulin conjugated to alkaline phosphatase (Dakopatts) diluted 1 in 3000.

The membranes were washed again and immersed in substrate (0.75 ml of 5-bromo-4-chloro-3-indolylphosphate, Sigma, 4 mg/ml in methanol: acetone 2:1 v:v; 5 ml of nitroblue tetrazolium (Sigma) 1 mg/ml; 0.2 ml of 1 M magnesium chloride; 44.05 ml of 0.1 M methanolamine-HCl buffer, pH 9.6) for 15 min; the reaction was then stopped by rinsing in water.

Immunoblotting of colonies

Suspensions of the type strains of *M. curtisii* were diluted and inoculated on to 10% HBA to give individual colonies after incubation for 48 h. Filter paper disks (Whatman) were then placed on each plate and left for a few minutes, removed and allowed to dry completely. The filters were treated with H₂O₂ 1% in methanol for 30 min to remove inherent bacterial peroxidase. After rinsing twice in distilled water, the filters were immersed in antibody diluted in washing buffer (as before) without the addition of BSA, for 2 h at 37°C on a rotary shaker. After a further three washes rabbit anti-mouse immunoglobulin conjugated to peroxidase (Dakopatts) diluted 1 in 3000 was added for 2 h as before. The filters were washed again and then immersed in substrate (3, 4, 3', 4'-tetra-aminobiphenyl hydrochloride, BDH, 0.5 mg/ml in 50 mM Tris-HCl, pH 7.4, containing H₂O₂ 0.01%).

Immunoelectronmicroscopy

Samples of the organisms in suspension were applied to grids previously coated with formvar/carbon, drained and allowed to dry before being treated with MAb and immunogold (goat anti-mouse IgG conjugated to 25-nm gold colloidal particles; Janssen Life Sciences Products) as follows. Grids were immersed in BSA 1% w/v in 20 mM Tris-buffered saline, pH 8.2 (BSA-TBS) for 10 min, drained and placed in tissue-culture supernate containing MAb for 2 h at room temperature. After thorough washing, grids were again placed in BSA-TBS, then in gold conjugate diluted 1 in 20 in BSA-TBS 0.1% w/v for 2 h at room temperature, followed by two more washes, first in 20 mM TBS then distilled water. Control grids were prepared with saline instead of the MAb, followed by labelling with gold conjugate as above. Grids were negatively stained with phosphotungstic acid and allowed to dry before being examined in a Philips 301 transmission electronmicroscope.

Results

Monoclonal antibodies

A series of seven MABs was produced after a single fusion of spleen cells from mice inoculated with each type strain. Four were chosen for further study on the basis of their reactivity when screened against various strains of *Mobiluncus* and their ability to react in immunofluorescence (IF) studies. The antibodies were designated MABs 179.1, 179.3, 180.1 and 181.1. The immunoglobulin subclasses of the antibodies were IgG2b (179.1) and IgG1 (179.3, 180.1 and 181.1).

Immunofluorescence

In IF studies, three of the four MABs (179.1, 179.3 and 180.1) showed activity against the type strains of both subspecies of *M. curtisii*, NCTC 11656 and 11657, but not against *M. mulieris* NCTC 11658. MAb 179.1 showed equal activity against both subspecies whereas MABs 179.3 and 180.1 showed activity against both subspecies but at different titres. MAb 179.3 showed a higher titre against *M. curtisii* subsp. *holmesii* whereas the titre of MAb 180.1 was higher against *M. curtisii* subsp. *curtisii*. The remaining MAB, 181.1, showed activity against *M. mulieris* NCTC 11658 but not against *M. curtisii* NCTC 11656 or NCTC 11657. The titres of all antibodies varied between different supernates but an example from twice cloned cells is shown in table I.

A range of clinical isolates was tested to determine the sensitivity of the MABs, i.e., the number of strains detected. MABs were used at a dilution of 1 in 4, which was chosen to detect species-specific antigens. All 37 strains of *M. curtisii* reacted with one or more of MABs 179.1, 179.3 and 180.1. Five patterns of reactivity were seen (table II). The

Table I. Titres of supernate against the type strains of *Mobiluncus* spp. in immunofluorescence (IF) tests

MAB	IF titres with		
	<i>M. curtisii</i>		
	subsp. <i>curtisii</i> NCTC 11656	subsp. <i>holmesii</i> NCTC 11657	<i>M. mulieris</i> NCTC 11658
179.1	128	128	0
179.3	16	32	0
180.1	512	128	0
181.1	0	0	16

Table II. Pattern of reactivity of clinical isolates of *M. curtisii* with MAb 179.1, 179.3 and 180.1

Pattern	Number of strains	Reactivity with MAb		
		179.1	179.3	180.1
A	16	+++	+++	+++
B	8	+++	—	+++
C	9	—	—	+++
D	3	+++	+++	—
E	1	—	+++	+++

majority of strains, 34 (92%) of 37, reacted with MAb 180.1. None of the clinical isolates of *M. curtisii* showed fluorescence with MAb 181.1.

Only 6 (24%) of the 25 clinical isolates of *M. mulieris* tested by IF gave positive reactions. However, even in positive smears only a proportion of cells fluoresced strongly, the remainder being weakly positive or negative. This phenomenon was also seen with the subspecies of *M. curtisii* and MABs 179.1, 179.3 and 180.1. None of the strains of *M. mulieris* reacted with MABs 179.1, 179.3 or 180.1.

The four antibodies were highly specific for the relevant species of *Mobiluncus* and did not react with any of the other bacterial genera tested.

Immunoblotting of SDS-PAGE

Immunoblotting was used to characterise the epitope(s) against which these antibodies were directed. The type strains were used in initial experiments and the clinical isolates were compared with these subsequently.

MABs 179.1 and 179.3 reacted with epitopes in *M. curtisii* NCTC 11656 and NCTC 11657 on a protein band that had an electrophoretic mobility corresponding to an Mr of 75 Kda in the absence of a reducing agent but of 82 Kda in its presence. The same pattern was seen with those clinical isolates that gave a positive IF reaction. IF-negative strains showed no band on immunoblotting.

MAB 180.1 reacted with an epitope in both *M. curtisii* type strains on a protein with a mobility corresponding to 75 Kda. The Mr was not affected by a reducing agent. With clinical isolates this antibody reacted with an epitope on a protein of 75 Kda or of 71 Kda or on both (fig. 1). When both proteins were reactive, the amount of epitope expressed by each protein was often unequal. The reaction of each MAB with clinical isolates of *M. curtisii* is summarised in table III.

MAB 181.1 reacted weakly with a protein of c. 90 Kda in *M. mulieris*, but only when the cell lysate was prepared in the absence of a reducing agent. The difficulty of detecting weak antibody

Fig. 1. Immunoblot of MAB 180.1 against strains of *M. curtisii*. Tracks 1–8, clinical isolates of *M. curtisii*; track 9, *M. curtisii* NCTC 11657; track 10, *M. curtisii* NCTC 11656.

Table III. Reaction with clinical isolates of *M. curtisii* by immunoblotting

MAb	Mr (Kda) of reactive protein determined by SDS-PAGE	
	with reducing agent	without reducing agent
179.1	+	—
	82	75
179.3	82	75
180.1	71	71
	75	75
	71/75	71/75

reactions was attributed to the relatively poor transfer of high-mol.-wt proteins in this system. Twelve of the 25 clinical isolates of *M. mulieris* expressed an epitope also on a protein of 90 Kda; six of these strains were IF positive and six were IF negative. The antibodies did not cross-react between species of *Mobiluncus*.

Immunoblotting of colonies

Immunoblotting was used to determine the degree of expression of the epitope detected by MAb 180.1 by individual colonies of populations of *M. curtisii* NCTC 11656 and NCTC 11657. Only a proportion of the colonies were labelled strongly; the remainder showed weak or no reactivity. Continued subculture reduced the number of colonies expressing the epitope but this was not a consistent finding.

Immunogold staining

The staining of the type strains by immunogold spheres showed that all four MAbs detected an epitope exposed on the cell surface (fig. 2). However, as was seen in IF and colony-blotting studies, each MAb detected only a subpopulation of cells. *M. curtisii* exhibited the epitope in a number of ways—bipolar, evenly distributed over the cell, or on one of two daughter cells (fig. 2).

Discussion

Motile anaerobic curved rods seen in vaginal secretions have proved difficult to isolate and purify because of their fastidious growth requirements and the presence of numerous other anaerobes in the vaginal secretions. We have raised a series of MAbs in an attempt to produce a rapid detection

system. To evaluate such reagents it is important to investigate both the sensitivity and specificity. We have tested the sensitivity with a range of organisms which we believe belong to the genus *Mobiluncus*. When used in combination, the three MAbs directed against *M. curtisii* showed 100% sensitivity and specificity. Of the 37 clinical strains of *M. curtisii* tested 26 (70%) reduced nitrate. Nitrate reduction and the ability to migrate through soft agar were the two criteria used by Spiegel and Roberts (1984) to distinguish between the subspecies of *M. curtisii*. Our results suggest that the majority of the strains were *M. curtisii* subsp. *holmesii* but as we did not test for the ability to migrate through soft agar we are unable to subspeciate confidently on the basis of a single criterion. There was no relationship between the pattern of reactivity with the three MAbs, 179.1, 179.3 and 180.1, and the reduction of nitrate, hence with the subspecies.

Of the individual MAbs, 180.1 reacted with 34 (92%) of the 37 strains and shows the greatest potential for use in diagnostic tests. The sensitivity of the individual antibodies may be higher when they are used directly on vaginal smears or on freshly isolated organisms. It is essential in characterisation studies such as these that the organisms are purified, therefore all isolates we have used have been subcultured many times. We have shown that MAb 180.1 reacts with an epitope which is detected only on a subpopulation of each strain and is lost on subculture, either by selection of non-expressing colonies or because expression has been switched off. This type of phase variation has been documented in several organisms including the loss of type-b capsule in *Haemophilus influenzae* (Hoiseth *et al.*, 1985) and loss of piliation in *N. gonorrhoeae* (Sparling *et al.*, 1986). It is also possible that the inability of the antibody to detect the epitope on some strains may be due to masking of the epitope by other outer-membrane material such as polysaccharide.

We have raised a single MAb with activity against the long curved rods, *M. mulieris*, and this reacted with only a small number of the clinical isolates tested. Although our strains were identified by the criteria described by Spiegel and Roberts (1984), the non-reactive strains may be atypical. Alternatively, antigenic heterogeneity in this species may limit the value of any single MAb.

The taxonomy of anaerobic curved rods and their inclusion in the genus *Mobiluncus* has been controversial, particularly the number of species and the separation of subspecies (Holst *et al.*, 1982; Christiansen *et al.*, 1984). Recently Vetere *et al.* (1987)

Fig. 2. Immunogold staining of *M. curtisii* subsp. *curtisii* NCTC 11656 with MAbs 179.1 (a), 179.3 (b) and 180.1 (c), and (d) of *M. mulieris* NCTC 11658 with MAb 181.1.

have performed a large number of tests on isolates of *Mobiluncus* spp. They have described four groups of strains—typical short and long forms and atypical short and long forms. They have distinguished these groups by their reaction with Gram's stain, metronidazole susceptibility, ability to hydrolyse arginine and hippurate, and the presence of β -galactosidase. We also have encountered motile anaerobic curved rods in clinical material which could not be identified as *Mobiluncus*. All of these were non-reactive with these MABs. However, it is not possible to determine whether any of the strains of *M. mulieris* that did not react with MAB 181.1, or our unclassified anaerobic curved rods, belong to the atypical groups described by Vetere *et al.* (1987) because we did not test for arginine hydrolysis.

If MABs are to be used in studies to elucidate the role of *Mobiluncus* spp. in bacterial vaginosis, it is probable that several antibodies will be required to detect both the long and short forms. Hallen *et al.* (1987) have shown that of 90 strains of *Mobiluncus* spp. isolated from vaginal specimens, 44% were *M. curtisii*, 34% *M. mulieris* and in 21% both strains occurred together.

The MABs we have raised react with epitopes which are exposed on the cell surface and appear to be subject to phase variation. The presence of the epitope detected by MAB 180.1 on surface-exposed

proteins with mobilities corresponding to two distinct Mr values of 71 and 75 Kda may be of interest in the pathogenicity of this organism. It is not known whether this represents the same epitope present on two different proteins or if it indicates a family of related proteins. In *N. gonorrhoeae*, the outer-membrane protein, PII, consists of a family of proteins which exhibit both phase and antigenic variation. These proteins are known to enhance the ability of gonococci to adhere to vaginal epithelial cells (Blake and Gotschlich, 1983). We now have preliminary results which show that MAB 180.1 inhibits the attachment of *M. curtisii* NCTC 11656 and 11657 to exfoliated vaginal epithelial cells *in vitro*. It is possible that this is an analogous situation to that of protein II of *N. gonorrhoeae* and that we have detected an outer-membrane protein family in *M. curtisii* that may have a role in colonisation.

We have raised MABs to *M. curtisii* that, if used together, could aid the detection of these organisms in genital specimens. We have only a single MAB to *M. mulieris* which does not appear to be sufficiently cross-reactive for diagnostic purposes. If the true incidence of *Mobiluncus* spp. in bacterial vaginosis and in normal women is to be determined, good detection systems will be necessary to overcome the problems of culture. Although DNA probes have shown promising results, MABs are more easily used in most laboratories.

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