Identification of Two New Antigenic Subgroups within the Genus *Mobiluncus*

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Received 25 March 1991/Accepted 27 June 1991

Classifications of 48 atypical clinical isolates of *Mobiluncus* spp. were determined on the basis of biochemical reactions, morphology, antigenic profiles, and DNA hybridization studies. Two new subgroups with unique antigenic profiles are described. Like typical *Mobiluncus* species, the antigenic variant of *M. mulieris* is associated with bacterial vaginosis. The atypical isolates of *M. curtisii* were frequently recovered from women with normal vaginal flora and were also recovered from sterile body sites. These isolates may be incorrectly identified if current biochemical and morphological criteria are used for identification. Gram stain morphology, however, correctly identifies these isolates to the species level. The characterization of these atypical isolates has important implications for future investigations in which serological methods are used for diagnosis, epidemiology, and determination of pathogenicity of *Mobiluncus* spp.

Members of the genus *Mobiluncus* are anaerobic curved rods which are associated with bacterial vaginosis (9). Bacterial vaginosis has been associated with chorioamnionitis and preterm delivery (5, 7), and it is possible that *Mobiluncus* species may play an important role in these processes. Furthermore, *Mobiluncus* species has recently been identified as a risk factor for postabortal pelvic inflammatory disease (12). Some evidence has suggested that vaginal colonization by *Mobiluncus* species is associated with increased vaginal bleeding, which is accompanied by a rise in antibody to *Mobiluncus* species, suggesting that there may be endometrial infection (11). Others have failed to find an association between *Mobiluncus* species and menstrual irregularities among women with bacterial vaginosis (6).

It has been difficult to determine the true prevalence of *Mobiluncus* species in various disease processes because of the difficulty in isolating this organism by using routine culture media. While fluorescence-labeled monoclonal antibodies could be used for the detection of *Mobiluncus* species (3), our earlier studies (17) have shown that 18% of clinical isolates are antigenically distinct from the type strains of *Mobiluncus* species and that the majority of these antigenically distinct strains fail to react with monoclonal antibodies specific for the type strains (17). The accurate detection of *Mobiluncus* species by serologic or antigen detection tests will require an understanding of all major antigenic subgroups.

The purpose of the present study was to characterize the antigenically atypical *Mobiluncus* species.

MATERIALS AND METHODS

Organisms. Forty-eight clinical isolates of *Mobiluncus* species were examined. These strains were selected from a larger group of isolates described previously (17) because of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) protein profile patterns which were atypi-

cal for their biochemically determined species. Strains were propagated and identified to the species and subspecies levels on the basis of gas chromatographic analysis of metabolic by-products, morphology, and motility as described previously (19). Strains which hydrolyzed hippurate, produced NH₄ from arginine, and were variable on Gram staining were identified as *M. curtisii*, whereas those which did not hydrolyze hippurate or produce NH₄ from arginine and were gram negative were identified as *M. mulieris*. Subspecies identification of *M. curtisii* was based on nitrate reduction (19). α -Galactosidase activity was measured by using a modification of the spot test described previously (2), with 4-methylumbelliferyl- α -D-galactopyranoside (Sigma Chemical Company, St. Louis, Mo.) used as a substrate.

Antisera and monoclonal antibodies. Polyvalent rabbit antisera were raised against each American Type Culture Collection (ATCC) type strain of *Mobiluncus* species as well as two clinical isolates (SLH-2, SLH-29) that were selected by protein profile analysis and that suggested new subgroups of *Mobiluncus*. Antisera were produced and collected as described previously (3, 17).

SDS-PAGE analysis. Whole organisms were harvested and processed as described previously (17). Briefly, organisms were suspended in phosphate-buffered saline (PBS; pH 7.2) and solubilized in an equal volume of 0.1 M Tris (pH 6.8) containing 2% SDS, 2% 2-mercaptoethanol, and 20% glycerol. Specimens were electrophoresed at 50 V for 16 to 18 h at room temperature on 10% acrylamide gels as described by Laemmli (10).

Immunoblots. Proteins were electrophoretically transferred from SDS-polyacrylamide gels to nitrocellulose paper as described previously (17). Briefly, the blots were blocked with PBS-0.5% Tween 20. Polyvalent rabbit antisera raised against each ATCC strain and clinical isolates SLH-2 and SLH-29 were diluted 1:100 in PBS-0.5% Tween 20 and were incubated with the blots at room temperature for 1 h. After washing, the blots were incubated with ¹²⁵I-protein A (0.8 μ Ci/10 ml of PBS-0.5% Tween 20; DuPont, NEN Research Products, Boston, Mass.) at room temperature for 1 h. Following a final wash, blots were exposed to Cronex MRF 32 film with intensifying screens at -70°C for 18 to 24 h.

Immunofluorescence. Fresh organisms were diluted in PBS

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TABLE 1. Characteristics of typical and atypical *Mobiluncus* species^a

Species and group	Gram stain reaction	Length (μm)	Hippurate hydrolysis	α-Galactosidase	NH ₄ from arginine	Major protein bands (kDa) on SDS-PAGE	Immunofluorescence assay			
							Reactivity with:			
							Anti- <i>M. curtisii</i>	Anti- <i>M. mulieris</i>	Anti-SLH-2	Anti-SLH-29
<i>M. curtisii</i>										
Typical	V	1.7	+	+	+	80–82	+	–	–	–
Group SLH-29	–	1.3–1.7	V (6/22)	– (1/15)	V (19/22)	71	– (0/22)	– (0/22)	– (1/22)	+
<i>M. mulieris</i>										
Typical	–	2.9	–	–	–	92–99	–	+	+	±
Group SLH-2	–	2.5–3.0	V (1/9)	– (0/9)	– (0/9)	84	– (0/9)	– (0/9)	+	– (0/9)

^a V, variable, which is less than 90% positive or greater than 10% negative for the characteristic; +, positive; –, negative.

^b Values in parentheses are number positive or negative/total number tested.

and immediately fixed in 3% formalin for 15 min. Suspensions were then centrifuged, and the pellet was resuspended in M199 medium (Sigma). Organisms were then placed on multiple-well slides (Cel-line, Newfield, N.J.), air-dried, and fixed in 70% ethanol (17). A panel of polyvalent rabbit antisera was used to stain each isolate. Antisera were diluted 1:50 (except for anti-SLH2, which was diluted at 1:40) in PBS–1% bovine serum albumin. Following application of the antibodies, the slides were incubated for 30 min at 37°C in a humidified chamber. Slides were washed three times in PBS and once in distilled H₂O. Slides were incubated with fluorescein-conjugated sheep anti-rabbit immunoglobulin G (Sigma) diluted 1:1,000 in PBS–2% Tween 80. Slides were washed and covered with coverslips as described previously (17). The degree of fluorescence was graded from negative to 4+.

Dot blot hybridization. Whole chromosomal DNA dot blots were prepared from one blood agar plate of *Mobiluncus* species grown and lysed as described previously (15). Dot blots of the bacteria were prepared on nitrocellulose and hybridized with nick-translated whole chromosomal DNA probes from *M. curtisii* ATCC 35241 and *M. mulieris* ATCC 35243, which were prepared as described previously (19). Hybridization was conducted overnight at 42°C under stringent conditions of 50% (vol/vol) formamide, 0.1% (wt/vol) polyvinylpyrrolidone, 0.1% (wt/vol) albumin, 0.1% Ficoll, 0.1% (wt/vol) SDS, 0.05 M monobasic sodium phosphate (pH 7.4), 0.005 M EDTA, 0.76 M NaCl, and 100 μg of boiled calf thymus DNA per ml. The filters were then washed three times for 10 min each time at 52°C with 0.015 M NaCl–0.0015 M sodium citrate (16). Known positive controls were included in each set. Those strains which did not hybridize with either probe were grown in broth, and DNA was prepared as described previously (19). The pure DNA was then retested with the two DNA probes. Isolates with ≥80% DNA homology with the whole chromosomal DNA from the type strain were considered to be the same species.

RESULTS

Correlation of DNA probe with biochemical identification. Forty-eight clinical isolates of *Mobiluncus* were selected for investigation because their protein profiles on SDS-PAGE were atypical for their biochemically determined species. Each strain was independently classified by DNA hybridization, Gram stain morphology (short versus long), biochemical reactions, SDS-PAGE protein profile pattern, and reactivity with polyvalent antisera by both immunoblot and

immunofluorescence assays. Greater than 80% homology with DNA from the type strains of *Mobiluncus* was considered as the definitive means for identification of the species.

Comparison of DNA hybridization with biochemical identification within this group of isolates revealed discrepant results in 21 of 48 (44%) isolates. This contrasts with a discordance rate of 5% for those strains with typical protein profiles (data not shown). Eighteen isolates which were identified as *M. curtisii* by DNA hybridization failed to hydrolyze hippurate and would have been misidentified by biochemical methods. Two isolates were identified as *M. curtisii* on the basis of hippurate hydrolysis, but they reacted as *M. mulieris* with the DNA probe and were gram negative. One of the 48 strains was nonreactive with any DNA probe tested. This strain did not hydrolyze hippurate and had morphology typical of that of *M. curtisii*. For the remainder of the strains with atypical protein profiles (27 of 48, 56%), DNA probe results matched those of their biochemically determined species, as did their Gram stain morphology results (17 *M. curtisii* and 10 *M. mulieris*).

Identification of new antigenic groups. Among the 48 isolates, two groups emerged which possessed patterns of antigenic reactivity which were distinct from those of the ATCC strains and from those of other clinical isolates. The first of these groups was represented by the strain SLH-2 (Table 1). These were long (2.5 to 3.0 μm), gram-negative rods which hybridized with the whole chromosomal DNA from *M. mulieris*. This strain failed to produce NH₄ from arginine, did not hydrolyze hippurate, and was negative for α-galactosidase. However, this strain differed from most isolates of *M. mulieris* in that it possessed a unique protein profile, with its major protein band being located at 84 kDa, whereas the major protein bands of ATCC 35243 (*M. mulieris*) were located at 92 to 99 kDa (Fig. 1). Additionally, this strain was nonreactive in the immunofluorescence assay with the standard panel of monoclonal antibodies to the type strains of *Mobiluncus* described previously (3).

Eight additional strains were found to share these characteristics with SLH-2, with the exception of one strain which did hydrolyze hippurate (Table 1). These strains accounted for 9 of 48 (19%) of the atypical strains. Protein profiles of representative strains are shown in Fig. 1. These strains were further investigated in immunoblot and immunofluorescence assays with polyvalent antisera to SLH-2 and SLH-29 (representative of the second major antigenic group to be discussed below). Each strain showed strong homologous reactions with SLH-2 but negative heterologous reactions with SLH-29 (Fig. 2). Three additional atypical strains

FIG. 1. SDS-PAGE profiles of *Mobiluncus* isolates. Lane 1, *M. curtisii* subsp. *curtisii* ATCC 35241; lane 2, *M. mulieris* ATCC 35243; lanes 3 through 7, representative isolates of the SLH-2 group; lanes 8 through 12, representative isolates of the SLH-29 group; lane 13, *M. curtisii* subsp. *holmesii* ATCC 35242. Molecular weight markers (10^3) are on the left.

were identified as *M. mulieris* by DNA probe analysis. These isolates had antigenic cross-reactivities with SLH-2 and SLH-29.

The second antigenically distinct group was represented by the strain SLH-29. This isolate did not hydrolyze hippurate or produce α -galactosidase, but did produce NH_4 from arginine, and so it does not conform to the biochemical pattern for either *M. mulieris* or *M. curtisii* (Table 1). Its morphology was that of a very short (1.3- μm) curved rod, and it hybridized only with the DNA from *M. curtisii*. This strain was nonreactive in the standard panel of monoclonal antibodies. Its protein profile on SDS-PAGE was not typical

FIG. 2. Immunoblot analysis of *Mobiluncus* isolates with SLH-2 antiserum. Lane 1, *M. curtisii* ATCC 35241; lane 2, *M. mulieris* ATCC 35243; lanes 3 through 10, representative strains of the SLH-2 group; lane 11, SLH-29. Molecular weight markers (10^3) are on the left.

FIG. 3. Immunoblot analysis of *Mobiluncus* isolates with SLH-29 antiserum. Lanes 1 and 2, *M. curtisii* ATCC 35241 and *M. mulieris* ATCC 35243, respectively. These strains reacted weakly, but this is poorly visualized in this photograph. Lanes 3 through 8, representative strains of the SLH-29 group; lane 9, SLH-2. Molecular weight markers (10^3) are on the left.

of that of either *M. mulieris* or *M. curtisii*, because its major protein band was located at 71 kDa. Twenty-one additional isolates had strong homologous reactions in immunoblot and immunofluorescence assays with polyvalent antisera to SLH-29 but negative heterologous reactions with SLH-2 antiserum (Fig. 3). There was heterogeneity among this group with respect to biochemical reactions, morphology, and SDS-PAGE profiles (Table 1).

Weak antigenic cross-reactivity was found in the immunoblot assay between these two distinct antigenic groups and the ATCC type strains of *Mobiluncus* (Fig. 2 and 3). *M. curtisii* ATCC 35241 failed to react with either anti-SLH-2 or anti-SLH-29. *M. mulieris* ATCC 35243 reacted strongly in the immunofluorescence assay with anti-SLH-2 and had a weaker reaction with anti-SLH-29.

There were 17 atypical isolates which did not belong to either group SLH-2 or SLH-29. Of these, 14 hybridized with the DNA from *M. curtisii*, and 3 isolates were identified as *M. mulieris* by DNA hybridization. Of the 17 isolates, 12 reacted with the monoclonal antibodies to the type strains, 2 reacted by the immunofluorescence assay with anti-SLH-2 or anti-SLH-29, and three were completely nonreactive by the immunofluorescence assay or Western blot (immunoblot). Thus, use of antibodies against SLH-2 and SLH-29, in addition to those against the type strains of *Mobiluncus*, detected 45 (94%) of 48 antigenically atypical isolates.

Source of isolates. In the SLH-2 group of *M. mulieris*, eight of the nine strains were isolated from vaginal specimens of women with bacterial vaginosis. The remaining strain was from an endometrial biopsy specimen of a woman with bacterial vaginosis. In the SLH-29 group, 16 of 22 (73%) vaginal isolates were from women with normal vaginal flora, whereas only 3 of 22 (14%) were from women with bacterial vaginosis. The remaining isolates were from semen, cul-de-sac fluid, and the chorioamnion.

DISCUSSION

BV is the most common cause of vaginal discharge (14). This syndrome has also been implicated in complications of pregnancy such as preterm labor, chorioamnionitis, amnionitis, and postpartum endometritis (5, 7, 21). In this study, we

included isolates of *Mobiluncus* isolated from the peritoneal fluid of a woman with pelvic inflammatory disease and the blood of a woman with cervical carcinoma. The actual prevalence of *Mobiluncus* species in clinical specimens is undoubtedly underestimated because of the difficulty encountered in isolating it from mixed anaerobic cultures and its stringent growth requirements. The data from this study also suggest that some isolates may not be correctly identified if only biochemical identification methods are used.

The antigenic profiles of the ATCC type strains of *Mobiluncus* have been described previously by our laboratory and others (1, 17, 18). The majority of clinical isolates share major proteins with type strains of one or both of the species; however, 18% of isolates previously described possessed atypical protein profiles on SDS-PAGE, and 57% of these failed to react with a panel of monoclonal antibodies (17). It is these atypical *Mobiluncus* strains which were the subject of the present investigation.

Atypical strains of *Mobiluncus* have been mentioned in other published reports. Vetere et al. (20) described four strains of *Mobiluncus* as atypical short forms and four strains as atypical long forms within a group of 33 isolates. These atypical strains were noted to have biochemical reactions which did not correspond to their classification by Gram stain morphology. They also described a strain of *M. mulieris* which had an SDS-PAGE profile typical of that of *M. curtisii* and concluded that the taxonomy of *Mobiluncus* species remained unclear.

Påhlson et al. (13) also described five atypical strains within their collection of 180 isolates. These strains were morphologically similar to *M. curtisii* but did not hydrolyze hippurate or react with monoclonal antibodies against *M. curtisii*. These strains had unique protein profiles on SDS-PAGE. Similar to our previous study (17), Garlind et al. (4) noted that 18% of *Mobiluncus* isolates examined were non-reactive with monoclonal antibodies.

We initially described clinical isolates of *Mobiluncus* as atypical if their protein profiles did not match those of their biochemically determined species. This is a fairly subjective method for classifying a strain as atypical, because there are no formal guidelines for distinguishing genuine differences from expected heterogeneity within a species. However, examination of these strains with combinations of morphological, biochemical, serological, and DNA hybridization techniques has shown that the majority of these atypical strains are indeed distinctive and that identifiable subgroups exist.

Hippurate hydrolysis has been suggested as a means of differentiating the species of *Mobiluncus*: *M. curtisii* hydrolyzes hippurate, while *M. mulieris* does not (19). Compared with the identification on DNA hybridization with the type strain, 42% of strains with atypical protein profiles had discrepant biochemical reactions. In contrast, 109 of 116 (94%) of *Mobiluncus* isolates with typical protein profiles reacted with the DNA probe of their biochemically designated species (data not shown). Thus, hippurate-negative *M. curtisii* isolates more commonly have atypical protein profiles. α -Galactosidase activity might be considered a differential test since most typical *M. curtisii* isolates produce α -galactosidase, while typical *M. mulieris* are negative for this enzyme (8). However, hippurate-negative *M. curtisii* is also negative for α -galactosidase (Table 1). Thus, there are no biochemical tests which reliably distinguish atypical *M. curtisii* from *M. mulieris*.

The major antigenic proteins within a group of typical *Mobiluncus* isolates have been described previously (17).

This study has demonstrated two additional antigenic subgroups within the genus that are represented by strains SLH-2 and SLH-29. Assuming that $\geq 80\%$ DNA hybridization with the whole chromosomal DNA from the type strains is the most reliable means of comparing relatedness at the species level, the antigenic profiles of SLH-2 and SLH-29 should be compared with those of the type strains of *M. mulieris* and *M. curtisii*, respectively. Both SLH-2 and SLH-29 failed to react with a panel of monoclonal antibodies raised against *M. curtisii* ATCC 35241 and *M. mulieris* ATCC 35243. However, homologous polyvalent antisera raised against these two strains was able to identify 44 of 48 (95%) atypical isolates.

In contrast to all of the clinical isolates in group SLH-2 which were from patients with bacterial vaginosis, the majority of strains in group SLH-29 were isolated from women with normal vaginal flora or from normally sterile sites (semen, cul-de-sac, chorioamnion). Thus, 86% of SLH-29 group isolates were from atypical sites, whereas only 12% of isolates with typical protein profiles were from atypical sites (data not shown). This finding, in conjunction with unique antigenic profiles, suggests that atypical *M. curtisii* is not typically associated with bacterial vaginosis but that it can invade the upper genital tract and cause disease.

In summary, we examined 48 clinical isolates of *Mobiluncus* with atypical protein profiles. Within this group we identified two major subgroups with unique antigenic profiles. Use of hippurate hydrolysis as a primary differential test for species identification is inadequate, and classification by length, as assessed by Gram staining, is the most accurate means of identification to the species level. Future serological investigation of members of this genus should include the major antigens from these two additional subgroups, in addition to those of the current ATCC type strains.

ACKNOWLEDGMENTS

This work was supported by research grant HD-AI 22192 and Sexually Transmitted Diseases/AIDS research training grant AI07140-12 from the National Institutes of Health and Public Health Service grant AI 24136-4.

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