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Isolation of *Mobiluncus* Species from Clinical Specimens by Using Cold Enrichment and Selective Media

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New and selective Rlk and SA media, combined with cold enrichment at 4 to 5°C, allowed isolation of *Mobiluncus* species from patients with bacterial vaginosis at higher rates than with conventional cultivation methods. Rlk medium consists of Columbia CNA agar supplemented with peptone, yeast extract, 5% laked rabbit or sheep blood, nalidixic acid, and tinidazole. SA medium consists of Columbia CNA agar supplemented with 2% rabbit serum, 1.6% laked rabbit or sheep blood, nalidixic acid, and tinidazole. Use of these selective media plus the cold enrichment technique permitted *Mobiluncus* species to propagate at rates similar to those of other anaerobic members of the vaginal flora.

Although anaerobic, curved, motile, gram-variable rods were observed in vaginal fluid as early as 1895, Curtis (2) in 1913 was the first investigator to isolate them from the female genital tract and culture them in the laboratory. Recent studies (10, 12–14) indicate that *Mobiluncus* species and perhaps other anaerobic bacteria, along with *Gardnerella vaginalis*, may be associated with bacterial vaginosis, a syndrome characterized by a radical change in the normal flora. Even though *Mobiluncus* species are unique and frequently observed in wet mounts or Gram stains of vaginal fluid of patients with bacterial vaginosis, the fastidious nature of these organisms has greatly impeded their recovery form vaginal fluid by conventional culture techniques (4, 6, 7, 10, 13, 14). The aim of the present study, therefore, was to develop a method that would improve the isolation of *Mobiluncus* species.

MATERIALS AND METHODS

Study population. Vaginal specimens, which were not sequentially numbered, were collected from 201 female patients, 78 attending Student Health Services at San Diego State University (SDSU) and 123 attending the Department of Obstetrics and Gynecology at the San Diego or La Mesa, Calif., Sharp Rees-Stealy Medical Center.

Methodology of specimen collection. Specimen collections at the two institutions (SDSU Student Health Services and Sharp Rees-Stealy) were not conducted simultaneously. After the processing of 78 specimens obtained from SDSU Student Health Services, the methodology of collection was evaluated, improvements were implemented, and collection of the Sharp Rees-Stealy specimens began. In both studies, vaginal fluid was collected from the lateral and posterior vaginal fornices with a cotton-tipped swab. The swab was inserted into anaerobic transport medium (Anaerobe Systems, Santa Clara, Calif.) such that the tip of the swab barely entered the agar (5 mm or less). The time and date of specimen collection were recorded.

Collection of SDSU Student Health Services specimens. After collection, the transport tube was taken to the laboratory (a process that took 10 min to 2 h) and placed in a refrigerator at 10 to 12°C until the swabs were streaked on Rlk, SA, brucella blood agar, and Mobi agar (C. A. Spiegel and K. J. Krueger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, C334, p. 172) for isolation of *Mobiluncus* spp. and one medium for isolation of *G. vaginalis*. The order in which the plates were inoculated was varied. All plates used to isolate *Mobiluncus* spp. were incubated in an anaerobic chamber (Anaerobe Systems) at 37°C for 48 h.

Collection of Sharp Rees-Stealy specimens. After collection, the transport tube was immediately placed in an ice chest (Little Playmate; Igloo Corp., Houston, Tex.) at 4 to 5°C. Specimens collected before noon were streaked on Rlk and SA media on the same day. Specimens obtained in the afternoon were placed in an ice chest at 4 to 5°C and either plated on the same day or refrigerated at 4 to 5°C until the following day. The time and date of inoculation were recorded. Each swab was used to inoculate two media (SA and Rlk) for isolation of *Mobiluncus* spp. and one medium for isolation of *G. vaginalis*. The procedure for inoculation and incubation of the media was the same as for the SDSU specimens.

Definition of bacterial vaginosis. Patients were diagnosed as having bacterial vaginosis if three of the following four characteristics were present (1, 3, 11): (i) thin, homogeneous discharge, (ii) pH greater than 4.5, (iii) clue cells, (iv) a fishy odor on addition of 10% KOH. All specimens were observed for clue cells and curved rods by wet mount and Gram stain. The pH was measured with pH paper (Micro Essential Laboratory, Brooklyn, N.Y.).

Immunofluorescence. Monoclonal antibodies for *Mobiluncus curtisii* and *M. mulieris* were provided by C. Pahlson, University of Uppsala, Uppsala, Sweden. Indirect immunofluorescence of vaginal secretions was performed by the procedure described by Pahlson et al. (9).

Bacterial strains. Six reference strains were studied for growth on SA and Rlk media, brucella blood agar, and Mobi agar, as well as for determination of the usefulness of room temperature versus cold enrichment specimen processing. Carol Spiegel, Clinical Laboratories, Madison, Wis., provided three strains. The remaining three strains were provided by Sydney Finegold, Wadsworth Veterans Administration Hospital, Los Angeles, Calif. *Bacteroides melaninogenicus, B. asaccharolyticus, G. vaginalis, Peptostreptococcus* species, and *Staphylococcus aureus* were obtained from the SDSU microbiology culture collection and used with the *Mobiluncus* spp. in determining the efficacy of cold enrichment.

Media. The titles of the media are not abbreviated and

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TABLE 1. Average colony size of reference strains and clinical isolates after 5 days of incubation[a]

Organism (type)[b]	Avg colony size (mm) on:					
	Rlk		SA		Brucella blood agar	Mobi agar
	With antibiotics	Without antibiotics	With antibiotics	Without antibiotics		
M. mulieris (R)	0.70	0.75	1.0	1.0	0.35	0.80
M. mulieris (C)	0.25	0.25	0.5	0.5	0.28	0.50
M. curtisii subsp. *curtisii* (R)	0.98	0.98	1.0	1.0	0.25	0.75
M. curtisii subsp. *holmesii* (R)	1.0	1.0	1.0	1.0	0.25	0.74
M. curtisii (C)	0.32	0.35	0.5	0.5	0.23	0.45

[a] Average size is based on media made with laked rabbit blood. Laked sheep blood requires 7 days to achieve the diameters indicated for rabbit blood.
[b] R, Reference strains; C, clinical isolates.

have no clinical significance; the initials were assigned abstractly. Rlk medium consisted of CNA agar (BBL Microbiology Systems, Cockeysville, Md.) with 0.6% yeast extract–2% peptone, with 5% laked rabbit or sheep blood–48 μg of tinidazole per ml–20 μg of nalidixic acid per ml added after autoclaving. SA medium was composed of CNA agar with 1.6% laked rabbit or sheep blood–2% rabbit serum–48 μg of tinidazole per ml–20 μg of nalidixic acid per ml added after autoclaving.

The plates were allowed to air dry for 2 days and then prereduced for 1 to 7 days before use, although we found that freshly prepared media or media 1 to 2 days old which had not been prereduced could be used without inhibiting the isolation of *Mobiluncus* spp. Plates could not be inserted into the anaerobic chamber directly after being poured, since they proved to be too moist for colony isolation.

Microbiological studies. All plates were examined visually for characteristic *Mobiluncus* colony morphology after 48 h. A Gram stain was then prepared from a section of the plate that indicated the presence of *Mobiluncus* spp. Reference strains, as well as fresh clinical specimens, were grown on Rlk and SA plates with and without the supplemented antibiotics, on brucella blood agar, and on Mobi agar. Maximum growth on the different types of media was determined after 5 to 7 days of incubation.

Biochemical identification. Identification of clinical and reference strains was based on criteria recommended by Spiegel and Roberts (15). Production of catalase was determined by methods described in the *Anaerobe Laboratory Manual* (5). Hippurate hydrolysis was determined as described by MacFaddin (8). Fermentation and nitrate reduction media (tryptic nitrate medium; Difco Laboratories, Detroit, Mich.) were supplemented with 2% rabbit serum and incubated in an anaerobic environment at 37°C for up to 5 days. Fermentation reactions were tested in peptone-yeast extract broth supplemented with either 0.5% glycogen, 0.5% melibiose, or 0.5% trehalose (5) and 1% bromcresol purple. A positive test was indicated by a yellowish green color change. All tests were inoculated with several colonies from a 5-day culture grown on either Rlk or SA medium without antibiotics. Uninoculated carbohydrate and nitrate media supplemented with 2% rabbit serum were incubated along with each panel and used as controls.

RESULTS

Mobiluncus colonies appeared on the different types of media after 48 h of incubation. The colonies were very small, convex, glossy, and translucent. The colony diameters after 5 to 7 days of incubation for both reference and clinical isolates are summarized in Table 1. The results of growth of pure *Mobiluncus* cultures and a mixture of *Mobiluncus* species, *G. vaginalis*, *Bacteroides* species, *S. aureus*, and *Peptostreptococcus* species after cold enrichment collection versus room temperature collection are shown in Table 2.

Of 123 clinical specimens from the Sharp Rees-Stealy Medical Center and 78 specimens from SDSU Student Health Services, 13 and 11, respectively, were positive for bacterial vaginosis and the presence of *Mobiluncus* spp. as indicated by curved rods in wet mounts, Gram stains, or both and immunofluorescence. Table 3 summarizes the room temperature-cold enrichment results of the SDSU Student Health Services specimens; seven specimens contained *M. mulieris* (long, curved rod), numbers 16, 20, and 42 also contained *Mobiluncus curtisii* (short, curved rod), and num-

TABLE 2. Results of growth of control organisms after room temperature or cold incubation in anaerobic transport medium

Organism and treatment	Growth of *Mobiluncus* spp. on:[a]			
	Brucella blood agar	Rlk	SA	Mobi agar
M. mulieris (reference strains)[b]				
Room temp				
1 h	2+	3+	3+	3+
5 h	2+	3+	3+	3+
24 h	1+	2+	2+	2+
Cold enrichment (5°C)				
1 h	1+	3+	3+	3+
5 h	1+	3+	3+	3+
24 h	1+	3+	3+	2+
Mixture[c]				
Room temp				
1 h	—	1+	1+	1+*
5 h	*	*	*	*
24 h	—	—	—	—
Cold enrichment (5°C)				
1 h	1+	2+	2+	*
5 h	1+	2+	2+	1+
24 h	—	1+	2+	1+*

[a] No. of colonies per plate: 1+, 1 to 9; 2+, 10 to 40; 3+, 45 to 100; *, *Mobiluncus* spp. present in small quantities but overgrowth by other organisms greatly impeded recovery. —, No growth.
[b] Similar results were obtained with *M. curtisii* (reference strains) and *M. curtisii* subsp. *holmesii* (reference strains).
[c] *Bacteroides* spp., *G. vaginalis*, *Peptostreptococcus* species, *S. aureus*, *M. mulieris*, *M. curtisii*, and *M. curtisii* subsp. *holmesii*.

TABLE 3. Results of room temperature and cold enrichment treatments (SDSU Student Health Services)

Specimen no.	Time (min) at room temp	Cold enrichment 10–12°C time (h)	Organism(s) detected by immunofluorescence	*Mobiluncus* detection by Gram stain of vaginal fluid[a]	Growth after room temp–cold enrichment treatment[b]		
					Rlk	SA	Mobi agar
8	30	4	*M. curtisii*	0–1+	—	—	—
10	40	3	*M. curtisii*	0–1+	—	—	—
16	15	4	*M. curtisii*; *M. mulieris*	3+	1+	2+	1+*
20	90	15	*M. curtisii*; *M. mulieris*	3+	—	—	—
25	30	3	*M. curtisii*	0–1+	—	—	—
28	90	7	*M. mulieris*	1–2+	—	—	—
30	35	0.66	*M. mulieris*	0–1+	—	—	—
39	120	4	*M. mulieris*	0–1+	—	—	—
42	20	6.5	*M. curtisii*; *M. mulieris*	0–1+	—	—	—
57	50	5	*M. curtisii*	3+	—	—	—
77	10	11	*M. mulieris*	0–1+	2+	1–2+	1+*

[a] Estimated number of *Mobiluncus* cells in vaginal fluid per oil field: 1+, 1 to 4; 2+, 5 to 10; 3+, 11 to 15.
[b] Number of colonies per plate: 1+, 1 to 9; 2+, 10 to 40. —, No growth. There was no growth on brucella blood agar. *, Overgrown by other organisms.

bers 8, 10, 25, and 57 contained only *M. curtisii*. The time factors involved, quantity of *Mobiluncus* sp. present in the specimen, and subsequent growth on the two selective media for the Sharp Rees-Stealy specimens are summarized in Table 4. Eleven specimens contained *M. mulieris*; numbers 67, 198, 137, and 201 also contained *M. curtisii* subsp. *holmesii*, and numbers 45 and 50 contained only *M. curtisii* subsp. *holmesii*.

DISCUSSION

Although the average colony diameters of the reference and clinical *Mobiluncus* strains differed greatly on the four types of media, addition of antibiotics to SA and Rlk media did not inhibit the growth of these organisms. The colony diameters both of the reference strains and of clinical *Mobiluncus* isolates were similar on Mobi agar, Rlk, and SA media (Table 1). However, when a mixture of organisms commonly found in bacterial vaginosis, including *Mobiluncus* spp., was collected on a swab, inserted into anaerobic transport medium, incubated at room temperature or 5°C, and then plated (Table 2), it was frequently impossible to locate the *Mobiluncus* spp. on Mobi agar. The lowest yield and the smallest colony size (Tables 1 and 2) of *Mobiluncus* spp. were obtained on brucella blood agar plates.

Even though the procedure for specimen processing was different for each institution (SDSU Student Health Services and Sharp Rees-Stealy), the most abundant growth of both *Mobiluncus* species was obtained on SA medium. In clinical isolation of *Mobiluncus* spp., SA and Rlk should be used in conjunction, since each medium allows the growth of different organisms because of its chemical composition (i.e., yeast extract, additional peptone, and laked blood versus serum and laked blood). Thus, if one type of medium is overgrown, the other usually is not. SA medium is the more inhibitory of the two, but it has little or no effect on gram-positive cocci.

Plates left in an aerobic environment for longer than 4 days and then reduced do not allow isolation of *Mobiluncus* spp. from clinical specimens and greatly diminish the growth of isolates in pure culture. Two Sharp Rees-Stealy clinical specimens (144 and 153) collected on the same day and then streaked on plates which were left for 4 days in an aerobic environment and then reduced for 1 week before use showed no *Mobiluncus* growth even though the organisms were present in the specimens in large numbers (Table 4).

The cold enrichment technique (Sharp Rees-Stealy specimens) helped immensely in *Mobiluncus* isolation. In over 50% of the cases (Table 4), the most abundant and almost

TABLE 4. Results of cold enrichment technique (Sharp Rees-Stealy)

Specimen no.	Total time (h) of cold enrichment	Organism(s) detected by immunofluorescence	*Mobiluncus* detection by Gram stain of vaginal fluid[a]	Growth after cold enrichment[b]	
				Rlk medium	SA medium
296[c]	1.5	*M. mulieris*	3+	1+	1+
277[c]	2.2	*M. mulieris*	3+	—	—
236	15	*M. mulieris*	3+	3+*	2+
234	15	*M. mulieris*	3+	2+	1+
201	16	*M. mulieris*; *M. curtisii*	0–1+	2+	2+*
198	21	*M. mulieris*; *M. curtisii*	4+	1+	3+*
48	1	*M. mulieris*	0–1+	2+	NA
45	14	*M. curtisii*	2+	—	1+*
50	14	*M. curtisii*	0–1+	1+	NA
67	2	*M. mulieris*; *M. curtisii*	4+	1+	4+*
144[d]	1	*M. mulieris*	0–1+	—	—
153[d]	15	*M. mulieris*	3+	—	—
137	2.5	*M. curtisii*; *M. mulieris*	3+	3+*	2+*

[a] Estimated number of *Mobiluncus* cells in vaginal fluid per oil field: 1+, 1 to 4; 2+, 5 to 10; 3+, 11 to 15; 4+, 16 to 20.
[b] Number of colonies per plate: 1+, 1 to 9; 2+, 10 to 40; 3+, 45 to 100; 4+, more than 100. *, *Mobiluncus* species were either the only organisms present or one of the major organisms on the plate. NA, Not available. —, No growth.
[c] There was a problem with the ice chest (see text).
[d] Plates not prereduced within the specified time (plates were dried for 4 days and then reduced for 1 week).

pure *Mobiluncus* growth occurred in specimens that were cooled immediately upon collection for 1.5 h or more at 4 to 5°C and then refrigerated at the same temperature for 13 h. This technique allowed *Mobiluncus* isolation even when the organisms were present in very small numbers. Room temperature specimen processing in combination with warmer cold enrichment (10 to 12°C) did not enhance *Mobiluncus* isolation (Table 3). Although immunofluorescence and Gram stain-wet mounts of vaginal fluid indicated the presence of *Mobiluncus* species, only 2 of the 11 specimens (no. 16 and 77) obtained from SDSU Student Health Services were positive by culture.

Room temperature was not conducive to isolation of *Mobiluncus* species in the presence of other members of the vaginal flora. This was demonstrated by the mixture control (Table 2), as well as by Sharp Rees-Stealy specimens 277 and 296. Both of these specimens were collected on the same day. A minor problem with the cooler on that day rendered the temperature very close to room temperature, with the transport tube being warm to the touch. Even though *Mobiluncus* sp. was present in large numbers in these samples, the specimen which was collected earliest and remained at room temperature for over 2 h had no *Mobiluncus* growth; the second specimen had very poor growth of the organism (Table 4).

Although previous studies (6, 7) have indicated that *Mobiluncus* sp. can survive for 2 to 6 h at room temperature before plating, the low isolation rates of these studies and the results with Sharp Rees-Stealy no. 277 and 296 plus the control study (Table 2) indicate that *Mobiluncus* sp. may be inhibited when in close proximity to certain members of the mixed flora present during bacterial vaginosis and on a swab with the *Mobiluncus* sp.

It appears that the cold enrichment technique slows down the metabolism of the competing flora to a rate that allows the *Mobiluncus* sp. to compete successfully with them. This, in combination with the selective plates, provides slow-growing and fastidious *Mobiluncus* spp. with the advantage they need to survive among the rapidly multiplying mixed flora.

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