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Characterization of *Gardnerella vaginalis* and *G. vaginalis*-Like Organisms from the Reproductive Tract of the Mare

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***Gardnerella vaginalis* has been isolated from women with bacterial vaginosis, from the genital tracts of asymptomatic women, and from several other infected body sites in humans. However, until recently, it has not been isolated from any other animal species. Between June 1988 and October 1989, 31 isolates identified as *G. vaginalis* and 70 isolates identified as *G. vaginalis*-like organisms have been recovered from the genital tracts of 93 mares from Michigan and Ohio. Identification was based on biochemical reactions, hemolysis on media containing blood from various animal sources, and susceptibility to select antimicrobial agents. This report details the characterization of *G. vaginalis* and *G. vaginalis*-like organism isolates obtained from the reproductive tracts of these mares and compares the equine isolates with human isolates.**

Gardnerella vaginalis was first described by Gardner and Dukes in 1955 after it was isolated from women with nonspecific vaginitis (3). Since that time, *G. vaginalis* has been isolated from many different human sources (10, 14, 16, 19) and there have been numerous reports describing the identification and characterization of *G. vaginalis* (2, 4, 5, 12, 17, 25). In the majority of these reports, identification of *G. vaginalis* was based on growth and biochemical characteristics. Our laboratory has recently isolated *G. vaginalis* and a *G. vaginalis*-like organism (GVLO) from cultures obtained from mares during routine reproductive soundness examinations and from mares with reproductive inefficiencies. This report characterizes these isolates on the basis of cellular and colonial morphology, biochemical reactions, susceptibility to select antimicrobial agents, hemolysis on media containing blood from various animals, and cellular fatty acid component profiles.

MATERIALS AND METHODS

Sampling group. Uterine culture specimens from 93 mares were collected from June 1988 to October 1989. These specimens were submitted for routine prebreeding and postfoaling soundness examinations, for investigation of chronic infertility, and for investigation of mares which had aborted. With the exception of one mare from Ohio, all of the mares were resident at horse farms located throughout Michigan. The mares ranged in age from 4 to 23 years. Each mare was restrained in stocks and prepared prior to specimen collection with a tail wrap and a perineal wash with cotton and a mild detergent or tamed iodine. In addition to the rectal sphincter and vulval lips, the wash extended to below the level of the clitoris and 10 to 15 cm to either side of the perineum. After the wash, each mare's perineal area was thoroughly rinsed with water.

Specimen collection and processing. Cultures were obtained by routine uterine culture procedures using sterile plastic sleeves, sterile lubricant, and a double-guarded equine uterine swab (VETKEM; Zoetcon Corp., Dallas, Tex.). The guarded end of the swab was inserted through the mare's vagina while protected in the palm of the gloved hand. At the cranial aspect of the vagina, the index finger was passed through the cervix to the internal cervical os. The guarded rod was pushed through the internal os to a site 2.5 cm past the os, at which point the internal sheath was advanced through the guard. The swab was then advanced to the point of contact with the endometrium (generally 2.5 cm) and was left in contact with the endometrium for approximately 30 s. The swab was then withdrawn into the inner sheath, the inner sheath was drawn back into the outer sheath, and the guarded culture instrument was removed from the mare's uterus. The specimens were transported to the laboratory in modified Stuart's transport medium (Marion Scientific, Kansas City, Mo.).

Submitted specimens were inoculated onto enriched blood agar (EBA) (tryptic soy agar base [Difco, Detroit, Mich.] supplemented with 5% defibrinated sheep blood [Cleveland Scientific, Bath, Ohio], 1% horse serum [BBL, Becton Dickinson, Cockeysville, Md.], and 1% yeast extract [GIBCO Laboratories, Lawrence, Mass.]), on phenyl ethanol agar (BBL) supplemented with 5% sheep blood, and on MacConkey agar (Difco). Specimens were also cultured in thioglycolate supplemented with 1% hemin (Sigma Chemical Co., St. Louis, Mo.) and 1% vitamin K (Sigma). The EBA and phenyl ethanol agar plates were incubated for 96 h at 35 to 37°C in a 5% CO_2 environment. The MacConkey agar and thioglycolate were incubated aerobically at ~5 to 37°C for 24 and 96 h, respectively.

Identification of *G. vaginalis*. Nonhemolytic colonies appearing on EBA after 48 h of incubation were subcultured onto EBA for further characterization. A commercial bacterial identification system (Rapid STREP bacterial identification system; Analytab Products, Inc., Plainview, N.Y.) was used to identify gram-negative to gram-variable bacilli that were catalase and oxidase negative. Additional tests, which have been used as a means of identifying *G. vaginalis* and GVLOs, included detection of hemolysis on media containing sheep, rabbit, or single- or bilayer human blood; susceptibility to metronidazole, sulfisoxazole, and sodium polyanetholesulfonate (SPS), and growth of alpha-hemolytic *Streptococcus* spp.; cellular fatty acid profiles; and electron microscopy. Enriched blood agar was used to detect hemo-

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TABLE 1. Type and occurrence of organisms isolated along with *G. vaginalis* (n = 24) and GVLOs (n = 61) from the equine reproductive tract

Organism	No. of times isolated
Alpha-hemolytic *Streptococcus* sp.	54
Corynebacterium sp.	34
Enterobacteriaceae	32
Staphylococcus sp.	16
Streptococcus zooepidemicus	13
Nonhemolytic *Streptococcus* sp.	4
Bacillus sp.	4
Streptococcus equisimilus	2
Pasteurella sp.	1
Actinobacillus equuli	1
Streptococcus equi	1
Micrococcus sp.	1
Cryptococcus laurentii	1

lysis of sheep blood. Tryptic soy agar base (Difco) supplemented with 5% defibrinated rabbit blood (Cleveland Scientific), 1% yeast extract (BBL), and 1% horse serum (GIBCO) was used to detect hemolysis of rabbit blood. Human blood-Tween (HBT) agar (BBL) was used to detect diffuse beta-hemolysis on bilayer human blood agar. Vaginalis agar (BBL) was used to detect diffuse beta-hemolysis on single-layer human blood agar. Susceptibility to select antimicrobial agents was determined as previously described (21). Susceptibility to SPS was determined by using brucella blood agar as described by Reimer and Reller (20).

Electron microscopy. Broth-grown cells were fixed in cold 0.1 M Na_2HPO_4-KH_2PO_4 (wt/vol)-buffered 2.5% glutaraldehyde at pH 7.2 and centrifuged. Cells were embedded in agar, washed in PO_4 buffer, and postfixed at room temperature in 1% OsO_4 in the above buffer. Following ethanol-propylene oxide dehydration, the agar cubes were embedded in Poly/bed 812 (Polysciences, Inc., Warrington, Pa.). Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM-10 electron microscope.

Cellular fatty acid analysis. Bacterial growth was harvested from one plate of brucella agar by adding 0.5 ml of sterile distilled water and then gently scraping the cells off the agar surface. The cells were transferred to a clean borosilicate screw-cap tube (20 by 150 mm) with a Teflon-lined screw cap. Fatty acids present in the bacterial cell walls were extracted for gas-liquid chromatography according to the procedure of Lambert and Moss (11). The extraction samples were analyzed for fatty acid methyl esters on a Varian 3500 gas-liquid chromatograph with a flame ionization detector by using a fused silica capillary column. The column oven was programmed to start at 150°C for 2 min. It was then ramped to 230°C at 4°C/min and was maintained at a temperature of 230°C for 6 min before being returned to the initial temperature of 150°C. The injector and detector temperatures were maintained at 250°C. Pressure was maintained at 12.0 lb/in^2 with a flow velocity of 33.2 cm/s and a split ratio of 50:1 (22).

RESULTS

Isolation of *G. vaginalis*. *G. vaginalis* and GVLO colonies appeared on EBA plates after 48 h of incubation as grayish white, opaque, domed, entire colonies less than 1 mm in diameter. A total of 31 *G. vaginalis* organisms and 70 GVLOs were isolated from the 93 mares from which specimens were cultured. Of the 31 *G. vaginalis* isolates, 7 were isolated in pure culture, and 9 of the 70 GVLO isolates were in pure culture. The remaining isolates of both organisms were recovered with as many as six other microorganisms (Table 1). Generally, when isolated in mixed culture, there were fewer than 50 CFU of *G. vaginalis* or GVLO along with other organisms (Table 2). The four most common organisms isolated with the *G. vaginalis* and GVLOs were alpha-hemolytic *Streptococcus* spp., *Corynebacterium* spp., *Staphylococcus* spp., and organisms belonging to the family *Enterobacteriaceae*.

TABLE 2. Quantities of *G. vaginalis* isolates, GVLOs, and other organisms isolated from mares

Organism	No. of isolates with CFU of:	
	<50	>50
Gardnerella vaginalis	20	11
GVLO	60	10
Alpha-hemolytic *Streptococcus* sp.	48	6
Corynebacterium spp.	28	6
Enterobacteriaceae	31	1
Staphylococcus sp.	15	1
Streptococcus zooepidemicus	10	3
Nonhemolytic *Streptococcus* sp.	3	1
Bacillus sp.	3	1
Streptococcus equisimilus	2	1
Pasteurella sp.	1	
Actinobacillus equuli	1	
Streptococcus equi	1	
Micrococcus sp.	1	
Cryptococcus laurentii	1	

Of the 93 mares, 16 were cultured more than once during the course of this study. Of these, GVLOs were isolated from eight on both primary and follow-up cultures. One mare yielded a GVLO on the initial culture and *G. vaginalis* on the follow-up culture. A third culture from that mare was negative for both organisms. Cultures from two mares grew a GVLO on primary isolation but were negative for both *G. vaginalis* and GVLO on subsequent cultures. For three mares, *G. vaginalis* and GVLOs were not detected on the initial cultures but GVLOs were present on subsequent cultures.

Identification of *G. vaginalis* and GVLOs was based on the isolation of pinpoint colonies on EBA that were gram-variable to gram-negative pleomorphic bacilli. These organisms were approximately 0.5 μm in diameter and 1.0 to 2.5

TABLE 3. Critical tests used for presumptive identification of *G. vaginalis*

Teest	% Positive reactions	
	Equine isolates	Human isolates[a]
Identification as gram-variable pleomorphic bacilli	100	100
Pinpoint growth on EBA at 48 h	100	100
Catalase reaction	0	0
Oxidase reaction	0	0
Hippurate hydrolysis	100	≥90
Beta-hemolysis on HBT	99	100
Gamma-hemolysis on sheep blood agar	100	100

[a] Reported previously (6, 13, 26).

TABLE 4. Summary of reactions of *G. vaginalis* and GVLO isolates obtained by using a commercial identification system

Reaction	% Positive reactions	
	G. vaginalis	GVLOs
Voges-Proskauer	0	0
Hippurate hydrolysis	100	100
Esculin hydrolysis	0	0
Pyrrolidonylarylamidase	0	0
α-Galactosidase	3	4
β-Glucuronidase	87	93
β-Galactosidase	39	46
Alkaline phosphatase	26	23
Leucine arylamidase	97	93
Arginine dehydrolase	0	0
Acdification		
Ribose	16	26
L-Arabinose	3	3
Mannitol	0	0
Sorbitol	0	0
Lactose	65	81
Trehalose	0	0
Inulin	0	0
Raffinose	0	0
Starch	32	13
Glycogen	6	0

μm in length. Additional criteria for the identification of *G. vaginalis* have included a lack of catalase and oxidase reactivity, a lack of hemolysis on sheep blood agar, diffuse beta-hemolysis on HBT agar, and hydrolysis of hippurate (Table 3). Commercial bacterial identification strips were used for biochemical characterization of the isolates, which demonstrated differences between the *G. vaginalis* and GVLO isolates. GVLO isolates were more likely to ferment ribose and lactose, whereas the *G. vaginalis* isolates were more likely to acidify starch (Table 4). Additional tests were used to further demonstrate these differences. For example, all of the isolates classified as *G. vaginalis* showed beta-hemolysis on rabbit blood and vaginalis agar, whereas only 67 and 37% of GVLO isolates were beta-hemolytic on these media, respectively (Table 5). In addition, all of the *G. vaginalis* isolates were resistant to the sulfisoxazole and susceptible to metronidazole and SPS. A total of 64% of the GVLO isolates were susceptible to sulfisoxazole, with 100 and 89% being resistant to SPS and metronidazole, respectively (Table 6). Electron microscopy of both organisms obtained from the mares (Fig. 1) showed the characteristic multilaminated cell wall configuration reported by Greenwood and Pickett for isolates from humans (5).

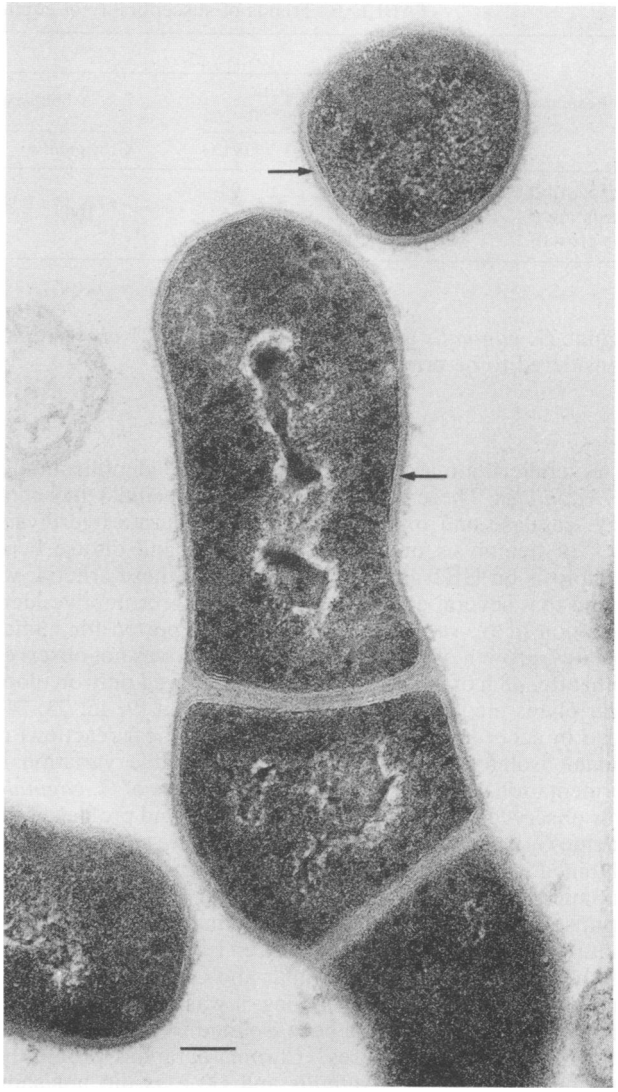

FIG. 1. Electron microscopy of equine *G. vaginalis*. Arrows show multilaminated cell walls. Bar, 0.1 μm.

Fatty acid profiles of equine *G. vaginalis* and GVLO isolates and human *G. vaginalis* isolates are shown in Table 7. While there were no major differences between the equine and human isolates, there was a minor difference at 16:0. The fatty acid profile of GVLO resembled the profile of the

TABLE 5. Results of hemolysis of equine *G. vaginalis* and GVLO isolates on media containing blood from various animals

Test	% Positive reactions on media with:							
	Sheep blood		Rabbit blood		V agar[a]		HBT agar[b]	
	G. vaginalis	GVLO	*G. vaginalis*	GVLO	*G. vaginalis*	GVLO	*G. vaginalis*	GVLO
Beta-hemolysis			100	67	100	37	100	98
Alpha-hemolysis				7				
Gamma-hemolysis	100	100		22		60		2
No growth				4		3		

[a] Vagar, vaginalis agar (a single-layer human blood agar).
[b] Bilayer human blood agar.

TABLE 6. Trends of susceptibility of equine *G. vaginalis* and GVLO isolates to various agents

Response	% Positive reactions to:							
	Alpha-hemolytic *Streptococcus* sp.		Sulfisoxazole		Metronidazole		SPS	
	G. vaginalis	GVLO	*G. vaginalis*	GVLO	*G. vaginalis*	GVLO	*G. vaginalis*	GVLO
Susceptibility	100	94		64	100	8	100	
Resistance		6	100	33		89		100
No growth				3		3		

equine *G. vaginalis* at 16:0. Differences at 18:1 *cis* were not considered to be critical.

DISCUSSION

Several criteria are recommended for the identification of *G. vaginalis*. These include colonial and cellular morphology, catalase and oxidase reactions, hippurate hydrolysis, lack of hemolysis on sheep blood agar, and diffuse beta-hemolysis on HBT agar (6, 13, 26). Using these criteria, we found that several equine isolates gave a presumptive identification of *G. vaginalis*. As has been reported for human isolates, growth of *G. vaginalis* or GVLOs was not observed until after 48 h of incubation and was observed only on blood agar plates incubated in a CO_2 environment (9, 15, 23, 24). Also in accordance with reported biochemical reactions of human isolates of *G. vaginalis*, considerable variation in fermentation reactions for the equine isolates of *G. vaginalis* was observed (1, 2, 4, 8, 17, 18, 24). Fatty acid profiles of the equine *G. vaginalis* and GVLO isolates were similar to those obtained for human isolates of *G. vaginalis*. Differences in antimicrobial agent susceptibilities and hemolytic activity against erythrocytes from different animal sources suggested that two different organisms had been isolated. However, on the basis of similarities in cellular and colonial morphology and in some biochemical reactions, it was concluded that *G. vaginalis* and a GVLO had been isolated from the reproductive tracts of these mares. Chromatographic profiles of human isolates of *G. vaginalis* and GVLOs did not show considerable variation and did not aid in their differentiation (1); this is also true for the equine isolates. In addition, the electron micrographs of the equine isolates demonstrated cell wall characteristics similar to those of human isolates as reported by Greenwood and Pickett (5).

The role of *G. vaginalis* or GVLOs as pathogens of the equine reproductive tract is yet to be determined. Analysis of reproductive histories of the mares at the time of culturing indicated that most of the mares were sampled during prebreeding soundness examination or postfoaling examination. During these examinations, there was no evidence of disease or history of reproductive inefficiencies. This suggests that, as in humans, these bacteria may be routinely isolated from the reproductive tract (7). There were, however, some mares with known reproductive inefficiencies including chronic infertility or recent abortions from which *G. vaginalis* and GVLOs were isolated in pure culture. It is not known whether these organisms contributed to the reproductive problems of the mares. Because *G. vaginalis* and GVLOs were isolated from the mares in very small quantities and usually with other organisms, their roles as pathogens are unclear. It could be that *G. vaginalis* and GVLOs do not play a role in a disease process but, rather, that they are normal inhabitants of the equine genital tract. In humans, other organisms such as *Mobiluncus* spp., *Bacteroides* spp., and *Mycoplasma hominis* have been isolated in association with *G. vaginalis* (7). In this study, cultures for these organisms were not performed; therefore, it is unknown whether they are part of the equine reproductive tract flora and whether they have a role in reproductive diseases in horses.

TABLE 7. Comparison of fatty acid profiles of human and equine *G. vaginalis* isolates and equine GVLO isolates

Organism	Gas-liquid chromatographic peak						
	12:0	14:0	16:1	16:0	18:1 *cis*	18:1 *trans*	18:0
Human *G. vaginalis*		4.4	2.0	54.8	27.3	0.4	10.4
Equine *G. vaginalis*	1.6	5.6	0.2	28.0	40.5		7.6
Equine GVLO	1.7	8.0		31.1	31.1	0.2	12.3

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