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Characterization and Comparative Genital Tract Pathogenicity of Bovine Mycoplasmas[1]

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Sixteen bovine genital mycoplasmal isolates obtained from semen and prepuce of bulls and from aborted fetuses were compared physiologically and serologically with the Donetta strain (tentatively *Mycoplasma agalactiae* var. *bovis*), a known pathogen. All isolates were distinct from the Donetta organism. Four appeared to be saprophytes, and the remainder were placed in one group which could not be further separated by the biochemical or serological methods used. Two of the organisms in the latter group have been subsequently identified as *M. bovigenitalium*. Uterine infusion of broth cultures of four isolates into virgin heifers failed to produce clinical evidence of disease, and significant lesions were not present at necropsy. The mycoplasmas were recovered from cervicovaginal mucus of only three heifers, and never for more than 3 days postinfusion. Since the organisms were not recovered from any organs at necropsy, it appears that the mycoplasmas were incapable of surviving in the clinically normal virgin female reproductive tract.

Since 1947, mycoplasmas have been considered as a possible cause of infertility in cattle (3). Up to the present time, two *Mycoplasma* species have been reported as capable of producing experimental lesions in the bovine female genital tract (1,6-8, 10). In this report, 16 mycoplasmal isolates from the bovine genital tract or aborted fetuses were compared, and the potential pathogenicity of four representative strains were tested in virgin heifers.

MATERIALS AND METHODS

The mycoplasmal organisms originated from three sources (Table 1). Thirteen of the isolates were from the semen or preputial secretion of bulls in an artificial breeding establishment in Connecticut (4), two were from aborted bovine fetuses from Connecticut (fetal isolates, Agway and Krogh, isolated and made available by L. F. Williams, Department of Animal Disease, University of Connecticut, Storrs), and one was from an aborted fetus from Vermont (Vermont-1 isolate received from W. Bolton, University of Vermont, Burlington). The pathogenic Donetta strain, used for comparative purposes, was that which had been isolated from a natural case of bovine mastitis and tentatively named *M. agalactiae* var. *bovis* (5). The media and methods used for maintenance of the isolates were similar to those previously reported (4, 9).

All mycoplasma isolates were studied with respect to growth at ambient temperature, at 37 C, and in the presence and absence of serum. Fermentation tests were made with 0.5% glucose and maltose as substrates and 0.0025% phenol red as indicator. In the methylene blue reduction test, quantities of 0.2, 0.6, and 0.8 ml of a 1:10,000 dilution of methylene blue were added to 5.0 ml of broth.

Antiserum to each strain was prepared in rabbits by a series of intravenous injections of washed antigen on days, 1, 2, 5, and 15. Blood was collected 10 days after the final injection of antigen. Fluorescent-antibody tests, with labeled Donetta antiserum (E. Karbe, Ph.D. Thesis, Univ. of Connecticut, Storrs, 1967), were performed (with the aid of E. Karbe, Department of Animal Diseases, University of Connecticut, Storrs) against each isolate.

The four strains selected for uterine infusion into heifers were Admiral, Acme, Krogh, and Vermont-1. These infusions consisted of 2.0 ml of 72-hr low-passage, frozen broth cultures placed into the body of the uterus during clinical estrus. Each broth culture was tested for viability and concentration subsequent to uterine infusion. Control animals were infused with heat-killed broth suspensions (Table 2). Cervicovaginal mucus and jugular venous blood samples were taken at days 1, 3, 6, 9, and 12 and every fourth day thereafter until the animals were necropsied (Table 2). At necropsy samples for cultural and histopathological examination were taken as previously described (8).

RESULTS

All 16 mycoplasmal isolates grew at 37 C, and 13 grew at 30 C. Three grew at 23 C and were the only ones that did not require serum enrichment. All were hemolytic and yeast extract usually increased the zone of hemolysis. Three mycoplasmal isolates readily fermented glucose and maltose when initially tested. These were the same mycoplasmas that grew at reduced temperature and

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without serum (Table 1). All isolates were capable of some degree of reduction of methylene blue.

The results of testing all antisera, by means of the tube agglutination test, against the Donetta, Admiral, Acme, Krogh, Vermont-1, and *M. laidlawii* antigens were as follows (Table 3). Donetta showed no significant reaction with any antiserum outside of the homologous system. The *M. laidlawii* antigen produced a strong agglutination with antiserum against the three non-serum-requiring isolates, a mild reaction with Maxim, Creator, and Ace, and negative aggluti-

TABLE 1. *Physiological characteristics of bovine genital mycoplasmas*[a]

Mycoplasmal isolate	Source	Requires serum	Growth at		Hemolysis	Methylene blue reduction[b]			Fermentations	
			22 C	37 C		0.2	0.6	0.8	Glucose	Maltose
Admiral	P	+	−	+	+	C	I	I	−	−
Lotus	P	+	−	+	+	I	I	I	−	−
Lee	P	+	−	+	+	C	I	I	−	−
Creator	P&S	+	−	+	+	I	I	−	−	−
Acme	P	−	+	+	+	C	I	I	+	+
Emory	P	+	−	+	+	C	I	I	−	−
Eric	P	+	−	+	+	C	I	I	−	−
Ace	P	+	−	+	+	I	I	−	−	−
Hominy	P	+	−	+	+	I	I	−	−	−
Mackayr	P&S	−	+	+	+	C	C	I	+[c]	+[c]
C.D.	S	+	−	+	+	I	I	−	−	−
Diadem	P	+	−	+	+	I	I	I	−	−
Maxim	P	+	−	+	+	I	−	−	−	−
Agway	F	+	−	+	+	I	I	−	−	−
Krogh	F	+	−	+	+	I	I	−	−	−
Vermont-1	F	−	+	+	+	C	C	I	+	+
Donetta	MG	+	−	+	+	C	C	I	−	−

[a] S, semen; P, prepuce; MG, mammary gland; F, fetus; I, incomplete; C, complete.
[b] Milliliter of 1/10,000 methylene blue reduced.
[c] Tendency to decolorize phenol red.

TABLE 2. *Clinical observations of heifers infused with mycoplasma*

Heifer accession no.	Mycoplasmal isolate	Necropsy at day	No. of mycoplasma infused	Returned to estrous	Recovery from vaginal mucus
H676	Admiral	7	2×10^8	NA[a]	D-1, D-3[b]
H672	Admiral	15	2×10^8	NA	−[c]
H674	Admiral	24	2×10^8	Yes	−
H673	Admiral	33	2×10^8	Yes	−
H687	Admiral	45	2×10^8	Yes	−
H677	Acme	7	$2 \times 10^{8.5}$	NA	−
H684	Acme	15	$2 \times 10^{8.5}$	NA	−
H685	Acme	24	$2 \times 10^{8.5}$	Yes	−
H675	Acme	33	$2 \times 10^{8.5}$	Yes	−
H682	Krogh	7	2×10^7	NA	−
H690	Krogh	15	2×10^7	NA	D-1[b]
H688	Krogh	24	2×10^7	Yes	−
H680	Krogh	33	2×10^7	Yes	−
H683	Vermont-1	7	$2 \times 10^{8.5}$	NA	−
H671	Vermont-1	15	$2 \times 10^{8.5}$	NA	−
H681	Vermont-1	24	$2 \times 10^{8.5}$	Yes	−
H678	Vermont-1	33	$2 \times 10^{8.5}$	Yes	−
H689	Control	15	Heat-killed	NA	−
H686	Control	33	Heat-killed	Yes	−

[a] Not applicable.
[b] Days postinfusion.
[c] Dashes indicate that no organisms were recovered at any time.

TABLE 3. *Serological relationships among the mycoplasmal isolates*

Antisera[a]	Antigens					
	Donetta	*M. laidlawii*	Admiral	Krogh	Acme	Vermont-1
Donetta	320	—[b]	—	—	—	—
M. laidlawii	—	320	—	—	80	10
Admiral	—	—	320	20	—	—
Lotus	—	—	40	320	—	—
Lee	—	—	80	40	—	—
Krogh	—	—	160	320	—	—
Agway	—	—	160	320	—	—
Mackayr	—	80	—	10	80	320
Maxim	—	—	320	10	10	—
Diadem	—	—	320	—	—	—
Creator	—	—	160	40	—	—
Acme	—	80	—	10	320	160
Emory	—	—	160	40	—	—
Eric	—	—	160	20	—	—
Ace	—	—	160	20	—	—
Hominy	—	—	20	20	—	—
C.D.	—	—	160	80	—	—
Vermont-1	—	160	—	10	320	320

[a] Highest serum dilution which produced a 3+ or stronger tube agglutination.
[b] Dashes indicate that a 3+ agglutination reaction was not obtained with any dilution.

nations with the rest. Admiral antigen had a negative reaction with Donetta antiserum, a negative reaction with the three non-serum-requiring isolates and *M. laidlawii*, and a moderate to strong agglutination with all antisera against the rest of the isolates except Hominy. Acme and Vermont-1 antigens were similar and gave a strong reaction with antisera against the three non-serum-requiring isolates and *M. laidlawii*, and minimal or negative reactions with the rest. The Krogh antigen produced a strong reaction with Agway and Lotus antisera, a moderate agglutination with antisera against Lee, Creator, and Emory, and a weak reaction with all of the rest of the serum-requiring isolates.

The fluorescent-antibody tests, with labeled Donetta antisera, gave negative results with all mycoplasmal strains except the homologous antigen.

The results of the uterine infusions of heifers were essentially negative (Table 2). No heifer exhibited a febrile response, and all animals which were kept for 21 days or longer returned to estrus. The mucus of estrus was clear and had no abnormal characteristics. Hemograms showed no significant abnormalities.

Cultures of cervicovaginal mucus were negative in all instances, with three exceptions. The day 1 and 3 samples from one heifer infused with Admiral and the day 1 sample of one heifer infused with Krogh were positive for the respective mycoplasma (Table 2). Mycoplasmas were not recovered from any organs and no gross lesions were seen at necropsy.

Histological changes were minimal or negative in all animals. A detailed study of the stratum compactum of the uterus revealed that considerable variation existed. This was true for the heifers which received the same mycoplasmal isolates and also for the control animals. The two primary cell types found in the stratum compactum were eosinophils and mast cells. There did not appear to be any meaningful correlation between the occurrence of eosinophils or mast cells, or both, and the particular isolate which was infused, or the number of days postinfusion.

DISCUSSION

The main purpose of the biochemical and serological tests was to determine how many different strains or species of mycoplasma were represented in the isolates. No specific attempt was made to identify them, other than to compare them serologically with the known Donetta pathogen [*M. agalactiae* var. *bovis* (5)] and the known saprophyte *M. laidlawii*. The results indicated that the 16 isolates represented both so-called saprophytic and nonsaprophytic organisms. Most investigators feel that the saprophytic mycoplasmas are not pathogenic. However, the isolation of a number of these strains from the oviduct of infertile cows has raised the question of their possible pathogenicity (11, 14). It was for these reasons that a "saprophytic type" of mycoplasma from

the bovine prepuce (Acme) and one from an aborted fetus (Vermont-1) were tested for pathogenicity in virgin heifers.

The agglutination tests indicated that at least two different groups of mycoplasmas were present among both the bull isolates and fetal isolates. The three non-serum-requiring mycoplasmas (Mackayr, Acme, and Vermont-1) showed almost no serological relationship to the rest of the isolates, but did appear serologically to be closely related to *M. laidlawii*.

The remainder of the preputial isolates appeared to fall into one large group which could not be further subdivided on the basis of the tube agglutination test. Two members of this group, Admiral and Krogh, have recently been identified by J. M. Al-Aubaidi, Cornell University, Ithaca, N.Y., as *M. bovigenitalium*.

The Donetta strain mycoplasma was distinct from all other isolates in agglutination and fluorescent-antibody tests. Many of the nonsaprophytic preputial and seminal mycoplasmal isolates which were used in this investigation may be either very similar or the same species of mycoplasma. This possibility has also been raised by other investigators (13; P. A. O'Berry, Ph.D. Thesis, Iowa State Univ., Ames, 1967).

The number of passages of the mycoplasma was purposely kept low to eliminate the possibility that numerous laboratory passages might decrease their virulence. The infused heifers did not exhibit any signs of disease at any time. The fact that none of the infused mycoplasmas could be recovered for more than 3 days postinfusion indicates that these mycoplasmas were unable to survive in the clinically normal genital tract of heifers. In contrast to this, the Donetta strain, which is capable of producing severe lesions, could be recovered for several months after uterine infusion (7, 8; P. A. O'Berry, Ph.D. Thesis, Iowa State Univ., Ames, 1967).

The experimental production of granular vulvovaginitis with *M. bovigenitalium* has been reported (1). In our study, this lesion was not observed in the heifers which were infused with the Admiral (preputial) and Krogh (fetal) organisms which appear to be strains of *M. bovigenitalium*. The reason for the absence of the vulvovaginitis was probably due to the fact that these mycoplasmal isolates were deposited in the uterus. In the previous report (1), it was necessary to mildly scrape the vaginal mucosa, before depositing *M. bovigenitalium*, to produce the vulvovaginitis experimentally.

Even though the strains of bovine genital mycoplasma used in this investigation failed to show any evidence of pathogenicity for the female genital tract, we should not make light of this group of organisms in regard to their role in bovine infertility. The potential to produce disease has been demonstrated by the pathogenicity of the Donetta strain organism (8, 9), and a mycoplasma from bovine semen has produced seminal vesiculitis, epididymitis, and mastitis (2). In man, a newly described T-strain mycoplasma has been recovered from a woman with spontaneous abortion (13), and T-strains have been recently isolated from the urogenital tract of cattle (15). There is little doubt that more investigation of these organisms is necessary to determine their proper place in the spectrum of genital tract pathogens.

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