

Microflora of the Cervico-Vaginal Mucus of Repeat Breeder Cows

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INTRODUCTION

Repeat breeding, like anestrus, is an economic loss in potential calf and milk production. Louca and Legates (14) have observed a decrease of approximately 72 kg of milk per month for calving intervals greater than 12 months during a period of three calvings and a loss of 0.08 calves per cow per year (20). The losses incurred by culling and selling nonproducing cows are no less negligible. In a study on Canadian dairy cow disposal, from 2.2 to 3.6% of all cows culled annually were due to breeding problems and the most frequent cause was failure to conceive after three or more services (3).

Limited studies have indicated that nonspecific bacterial infections with significant clinical manifestations or inapparent symptomatology are responsible for many cases of infertility in the cow. Studies on the microflora of the cervico-vaginal mucus, the cervix and anterior vagina of normal and repeat breeder cows have shown a wide spectrum of microflora (5, 15, 16). The organisms isolated include *Staphylococcus*, *Streptococcus*, *Escherichia coli*, *Corynebacterium*, *Haemophilus*, *Proteus* and *Bacillus* species. Less frequently, mycoplasmas and mycotic agents have been reported with doubtful significance.

The present study was conducted to assess the microflora (bacteria, fungi and mycoplasmas)

resident in the cervico-vaginal mucus of 72 normal fertile and 70 repeat breeder cows.

MATERIALS AND METHODS

Bacterial Flora

The cervico-vaginal mucus samples were drawn from both normal fertile and repeat breeder cows from 30 herds located in the south central region of Ontario during the period between May 1974 and January 1975. The cows were classified as repeat breeders in this study on the basis of the definition laid down by Gibbons (8). Pregnant animals and cows manifesting functional anestrus or cystic ovaries upon rectal palpation were not included in this study. The majority of the samples were collected from animals in the diestrus phase of the estrus cycle. No inseminations were recorded at least seven to eight days prior to sampling time. Vaginal examination with a speculum or any other manipulation of the reproductive tract via the vagina was avoided at sampling; hence, it was not possible to observe any inflammatory lesions (vaginitis) of the vaginal mucosa.

The cervico-vaginal mucus samples were collected by means of a sterile glass tube using negative pressure. The volume of mucus obtained from each cow was mixed with a measured volume of trypticase soy broth (TSB) to give a dilution of 1:10 (one part mucus to nine parts TSB) and transferred into sterile test tubes.

To obtain a wide spectrum of the flora in the cervico-vaginal mucus, several types of bacteriological media were required. The media used for the primary isolation of the microflora, the purpose for which they were employed and the incubation environments are listed in Table I. Each plate was inoculated with 0.04 ml of the diluted sample.

Plates incubated at 37°C were counted after 24 to 48 hours. The 24 hour counts were used as a check on the 48 hour counts recorded in this study.

Individually isolated colonies of the same morphotype were enumerated from appropriate

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TABLE I
 MEDIA AND CULTURAL ENVIRONMENTS USED FOR PRIMARY ISOLATION OF MICROORGANISMS
 FROM CERVICO-VAGINAL MUCUS OF COWS

Media	Purpose	Incubation 37°C	
		Environment	Period
Blood Agar ^a	Aerobes and Facultative Anaerobes	10% CO ₂ Tension	48 hours
MacConkey Agar ^a	Enumeration of Coliforms and Related Organisms	Aerobic	48 hours
Cystine Heart Agar	Enumeration of Campylobacter Species	85% Nitrogen 10% Carbon dioxide 5% Oxygen	5 days
Prereduced Blood Agar	Anaerobes	Anaerobic (Gas Pak) ^b	48 hours
Mead Agar ^b	Enumeration of Fecal Streptococci	Aerobic	48 hours
Mannitol Salt Agar ^a	Enumeration of Staphylococci	Aerobic	48 hours
Sabourauds Dextrose Agar ^a	Enumeration of Yeasts and Molds	Aerobic (room temperature)	12 days
PPLO Broth (or Agar) Base for growth of Mycoplasmas with additives		1. Aerobic 2. 10% CO ₂ tension 3. Anaerobic	48-72 hours

^aAll basic media were prepared from Difco dehydrated media, according to instructions from the manufacturer. Difco Laboratories, Detroit, Michigan.

^bBBL Division of Becton, Dickinson and Co., Canada Ltd., Clarkson, Ontario.

plates. All colonies including those of pinpoint size were counted from two to "too numerous to count" (TNTC). The numerical estimate of colonies per plate was multiplied by the dilution factor and the results recorded as the number of organisms per ml of mucus at 37°C. Distinctly isolated colonies were selected from appropriate plates, cloned and checked for purity of growth prior to characterization into respective genera and species. Characterization into respective genera and species were based on biochemical tests. The classification and specification of organisms was based on the schema presented in Bergey's Manual of Determinative Bacteriology (2). The statistical methods used were a chi-square test and a student *t* test according to the method of Remington and Schork (17).

Mycotic Flora

Individual isolated colonies were selected on the basis of a Gram stained smear and appearance of a wet mount preparation with lactophenol cotton blue. Representative isolates of yeasts and molds were identified and classified by the Laboratory Services Branch of the Ontario Ministry of Health, Toronto and the Clinical Microbiology Laboratory of the Ontario Veterinary College.

Mycoplasmas

The media used for the isolation of large colony mycoplasmas was prepared according to the method of Davis (4). Preparation of the media intended for the isolation of T-strain mycoplasmas¹ was according to the method of Gourlay (9). The large colony mycoplasmas were characterized by using specific antisera with the fluorescent antibody technique.

RESULTS

Bacterial Flora

One to six different types of bacteria were isolated, with plate counts of colonies ranging from two to "too numerous to count" (TNTC). A value of 120,000 (log 5.08) organisms per millilitre was assigned to counts designated TNTC for the purpose of analyzing the samples statistically. The value represented the largest number of colonies of any one species of bacteria enumerated on a single plate. In the normal fertile group, the number of cows yielding one, two, three, four and five types of isolates were five (6.9%), 23 (31.9%), 20 (27.8%), 19 (26.4%) and three (4.2%) respectively. One cow yielded a negative sample and one yielded a sample with six types of isolates.

In the repeat breeder group, the number of

¹Strains of T-mycoplasma are now called Ureaplasma.

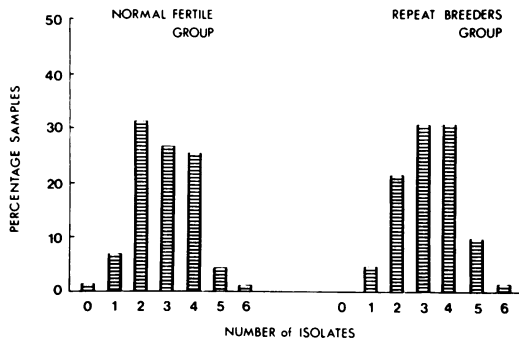


FIGURE 1. Number of bacterial isolates per sample of cervico-vaginal mucus from the normal fertile and repeat breeder cows.

cows yielding one, two, three, four and five types of isolates were three (4.3%), 15 (21.43%), 22 (31.4%), 22 (31.4%) and seven (10.0%), respectively. There were no negative samples and only one animal yielded six types of isolates (Figure 1).

The bacterial isolates were categorized into appropriate genera and species on the basis of recognized biochemical tests. Table II lists the diverse array of bacterial isolates from the two groups of animals. Of the bacterial isolates found, 22 of the 25 types were common to both groups of

animals. A single isolate of *Haemophilus somnus*, two isolates of *Staphylococcus aureus* and *Bacillus subtilis* in the repeat breeder group were the exceptions.

Organisms which occurred in high frequencies were grouped into their respective families for the purpose of a comparative statistical analysis of the two groups of samples. Table II shows the relative frequency of occurrence of the 25 species of bacterial isolates in the two groups of animals, including the respective total counts and the corresponding values expressed in logarithms. Table III indicates the corresponding values when the isolates were pooled into their respective families. The chi-square test conducted on the pooled frequency of occurrence of the five groups of microorganisms that occurred in relatively high frequencies in the two groups of animals gave an overall value of 6.5041. This χ^2 value revealed no significant difference in the frequency of occurrence of the five groups of organisms.

A further comparison was made between the geometric means of the five groups of organisms. A student *t* test on each group between the two sets of samples revealed a significant difference between the mean number of organisms in the two groups, Enterobacteriaceae and *Corynebac-*

TABLE II
RELATIVE FREQUENCY OF OCCURRENCE AND TOTAL COUNTS OF BACTERIA IN THE
NORMAL FERTILE AND REPEAT BREEDER COWS

Organism	Normal Fertile Group			Repeat Breeder Group		
	Frequency of Occurrence	Total Count	Log 10	Frequency of Occurrence	Total Count	Log 10
<i>Micrococcus</i> spp.	15	114,000	5.0569	20	162,000	5.2095
<i>S. aureus</i>	—	—	—	2	21,500	4.3324
<i>S. epidermidis</i>	19	186,000	5.2695	17	221,000	5.4333
<i>Str. bovis</i>	31	833,500	5.9209	25	826,000	5.9170
<i>Str. faecalis</i>	18	576,000	5.7604	18	861,500	5.9353
<i>Str. faecium</i>	5	254,000	5.4048	3	29,000	4.4624
<i>Str. acidominimus</i>	7	320,500	5.5058	7	228,500	5.3589
Unidentified Streps	11	120,000	5.0792	13	305,500	5.4850
<i>E. coli</i>	29	262,500	5.4192	23	653,000	5.8149
<i>P. vulgaris</i>	5	84,000	4.9243	—	—	—
<i>P. inconstans</i>	1	4,000	3.6021	1	6,000	3.7782
<i>K. pneumoniae</i>	1	5,500	3.7404	2	127,000	5.1038
<i>B. licheniformis</i>	17	66,500	4.8228	23	79,500	4.9004
<i>B. pumilus</i>	17	62,000	4.7924	20	115,500	5.0626
<i>B. subtilis</i>	—	—	—	2	8,000	3.9031
<i>B. firmus</i>	6	18,500	4.2672	7	34,500	4.5378
<i>C. pyogenes</i>	7	230,000	5.3617	16	910,000	5.9590
<i>Kurthia</i> spp.	6	29,000	4.4624	7	155,000	5.1903
<i>Neisseria</i> spp.	1	6,000	3.7782	1	4,000	3.6021
<i>B. catarrhalis</i>	4	28,500	4.4548	3	17,000	4.2304
Acinetobacter spp.	2	47,000	4.6721	2	6,500	3.8129
<i>P. multocida</i>	1	120,000	5.0792	2	140,000	5.1461
G+ pleomorphic anaerobes	2	26,000	4.4150	9	125,500	5.0986
G-Rods (N.I.)	1	11,000	4.0414	4	28,500	4.4548
<i>H. somnus</i>	—	—	—	1	120,000	5.0792

TABLE III

POOLED FREQUENCY OF OCCURRENCE AND TOTAL COUNT IN THE FIVE MAJOR GROUPS OF BACTERIA IN CERVICO-VAGINAL MUCUS OF FERTILE AND REPEAT BREEDER COWS

Group	Normal Fertile Group			Repeat Breeder Group		
	Frequency of Occurrence	Total Count	Log 10	Frequency of Occurrence	Total Count	Log 10
Micrococcaceae	34	300,000	5.4771	39	404,500	5.6069
Streptococceae	72	2,104,000	6.3230	66	2,250,500	6.3523
Enterobacteriaceae	36	356,000	5.5514	26	786,000	5.8954
Bacillaceae	40	202,500	5.3064	52	237,500	5.3757
<i>Corynebacterium</i>	7	230,000	5.3617	16	910,000	5.9590

terium pyogenes, respectively. There was no significant difference in the other three groups.

Mycotic Flora

In 142 samples examined for mycotic agents from both groups of animals, 20% possessed a flora. The samples were recorded as culturally negative when no mycotic growth was observed after 12 days of incubation.

A presumptive identification of the isolates on the basis of their typical morphology enabled the differentiation of the mycotic agents into yeast and molds. *Candida krusei* was the predominant isolate in both groups of animals. The remaining isolates included a single species of *Candida tropicalis* from the normal fertile group and a strain of *Cryptococcus albidus* from a specimen from the repeat breeder group. Upon comparing the relative proportion of animals that were positive for *Candida* and isolates resembling *Candida*, six (8.3%) of the cows from the normal fertile group were positive as compared to 12 (17%) of the cows from the repeat breeder group. Filamentous fungi were isolated from six (8.3%) of the normal fertile animals and six (8.6%) of the repeat breeder animals, respectively. The majority of the isolates from both groups were identified as *Penicillium* species along with species of *Alternaria*, *Cladosporium* and *Aspergillus*.

Four out of six animals that yielded fungi in the normal fertile group were culturally positive for both yeasts and molds, while only two animals in the repeat breeder group possessed a mixed mycotic flora.

Mycoplasmas

Thirty (21.4%) of the 142 samples were positive for T-strain mycoplasmas and 12 (8.5%) were positive for large colony mycoplasmas, respectively (Table IV). Only nine of the 12 samples that were positive for large colony mycoplasmas yielded colonies on reculture. All except one of these isolates were identified as *Mycoplasma bovis genitalium*. The single isolate was found to resemble *Acholeplasma laidlawii*.

In considering the results of each group of ani-

mals, four (5.6%) of the normal fertile animals yielded large colony mycoplasmas and 11 (15.3%) T-strain mycoplasmas, respectively. In the repeat breeder group, eight (11.4%) yielded large colony mycoplasmas and a comparatively higher number, 19 (27.2%) yielded T-strain mycoplasmas. Two animals in each group were positive for both T-strain and large colony mycoplasmas respectively. A chi-square test did not show a significant difference between the frequency of occurrence of the T-strain and large colony mycoplasmas in the two groups of animals.

DISCUSSION

The quantitative study of the organisms from both normal fertile and repeat breeder cows provides a means for comparison between the two sets of samples. In the present study, it is observed that 22 of the 25 types of bacterial isolates are common to both groups of animals. Single samples from both groups yield up to six different isolates. Decun and Rosu (5) found a mean of 1.3 species per sample from the cervical secretions. Heist and Tanabe (11) working with different parameters, recorded an overall mean of 1.9 microfloral types per heifer in their studies. In the present study, the mean number of different types of bacteria per sample are 2.89 and 3.26 for the normal fertile and repeat breeder cows, respectively.

Upon comparing each species of bacteria between the two groups of animals, it is observed that there is no remarkable difference in the frequency of occurrence of the different species. This signifies that there is a "normal" bacterial population resident in the cervico-vaginal mucus of both the normal fertile and repeat breeder cattle examined. Noteworthy, however, is the observation that a significant difference exists between the means of two of the major groups when submitted to the *t* test analysis. *Corynebacterium pyogenes* and organisms grouped under the family Enterobacteriaceae appear to show an increase in the repeat breeder group, although both

TABLE IV

MYCOPLASMA ISOLATES FROM THE CERVICO-VAGINAL MUCUS OF NORMAL FERTILE AND REPEAT BREEDER COWS

Sample No.	Normal Fertile Group		Sample No.	Repeat Breeder Group	
	T-strain	Large Mycoplasma		T-strain	Large Mycoplasma
WH/1	+	<i>M. bovis genitalium</i>	GV/1		<i>M. bovis genitalium</i>
MW/1	+	<i>M. bovis genitalium</i>	JG/2	+	<i>M. bovis genitalium</i>
HL/A/3	+		EBY/1		<i>M. bovis genitalium</i>
HL/A/4	+		EBY/3		<i>M. bovis genitalium</i>
HL/A/5	+		BON/3		<i>M. bovis genitalium</i>
NW/3	+		OW/1		Resembled <i>A. laidlawii</i>
ERS/A/8	+		MP/2	+	
ERS/B/6	+		SN/2	+	
ERS/C/8	+		HL/A/8	+	
ERS/C/9	+		HL/A/9	+	
OSB/2	+		DS/3	+	(ND)
AE/2		(ND)	DS/5	+	
SN/3		<i>M. bovis genitalium</i>	RD/1	+	
			EBY/2	+	
			EBY/5	+	(ND)
			EBY/6	+	
			ERS/A/1	+	
			ERS/D/3	+	
			ERS/D/6	+	
			NW/4	+	
			CG/1	+	
			CG/2	+	
			CG/6	+	
			CG/7	+	
Total	11	4		19	8

(ND) = Mycoplasma like colonies identified only on primary culture plates.

types of organisms occur in the normal fertile as well as the repeat breeder group to a comparable frequency.

Predominant among both groups are organisms of the genera *Micrococcus*, *Staphylococcus*, *Escherichia*, *Bacillus* and *Corynebacterium*. Organisms of the genera *Neisseria*, *Branhamella*, *Acinetobacter*, *Pasteurella*, *Klebsiella*, *Haemophilus*, *Proteus* and *Kurthia* constituted a minority group of isolates. Similar findings have been recorded by Easley *et al* (6), Gunter *et al* (10) and Martinez (15).

Gibbons *et al* (7) have observed that cultures from the infertile cows do not show a higher percentage of bacterial isolations than those from the fertile cows although the ratio of pathogens (26%) to nonpathogens (13%) is much higher in the infertile cows. Comparing the bacteriological findings, Nunn (16) observed that the genera of organisms isolated from the genital tracts of 41 random samples corresponded closely to the results obtained from 133 infertile cows. In explaining similar findings, Heist and Tanabe (11) stated, "Once microorganisms gain entrance into the uterus of repeat breeding cows, they may persist whether the animal returns to estrus or becomes pregnant". Such a persistence of certain bacterial types in repeat breeding cows has been observed by Easley *et al* (6) and Gunter *et al* (10). Sub-

sequent to the isolation of a wide spectrum of organisms from the vagina of cows with suboptimal breeding efficiency, Vigue *et al* (19) concluded that the vagina serves as a habitat for many types of saprophytic organisms, some of which are well known "opportunists" which under favourable circumstances, may impair fertility.

A distinctive feature is the finding that only a relatively low percentage (20%) of cows harbor a mycotic flora among both normal fertile and repeat breeders examined. The yeast forms occur more frequently than the molds. Apart from the single isolate of *Cryptococcus albidus*, *Candida* species and yeast cells resembling *Candida* are the common isolates in both groups of animals. The molds consisting of species of *Aspergillus*, *Penicillium*, *Cladosporium* and *Alternaria* are considered common constituents of the aerospora of cow barns.

Species of *Candida* and yeasts other than *Candida* have been incriminated as causative agents of vaginitis in human patients (18) and fungal infection has been associated with vulvovaginitis in sows (13). However, apart from the implication of certain species of fungi as potent abortifacients, the significance of mycotic agents in relation to the repeat breeder problem and infertility in cattle has not been documented.

In a total of 142 samples examined, 30 (21.1%) are positive for T-strain mycoplasmas and 12 (8.5%) are positive for large colony mycoplasmas, respectively. Anderson (1) has reported the isolation of T-strain mycoplasmas from nine and *Mycoplasma bovis* from two cows with a history of genital tract infection and infertility. On the other hand, Langford (12) has observed that 13% of T-strain mycoplasmas and 12% of *Mycoplasma bovis* were isolated from first and second calf healthy cows with normal breeding histories. Conclusive evidence as to the exact role that these organisms play in conception failures in cattle is yet lacking.

The findings of this study provide a basis for an impression about the three microfloral types, bacteria, fungi and mycoplasma in the reproductive tract of dairy cattle. Although these microorganisms are demonstrated in the cervico-vaginal mucus of both the normal fertile and repeat breeder cows sampled, it is not possible to evaluate their specific role in the repeat breeder problem of cows.

SUMMARY

The study furnished an assessment of the bacteria, fungi and mycoplasmas resident in the cervico-vaginal mucus of 72 normal fertile and 70 repeat breeder cows.

Qualitative studies enabled the characterization of 25 species of bacteria in the cervico-vaginal mucus from both groups of cows. The predominant isolates belonged to the genera *Streptococcus*, *Micrococcus*, *Staphylococcus*, *Escherichia*, *Bacillus* and *Corynebacterium*, respectively. The genera *Proteus*, *Klebsiella*, *Pasteurella*, *Neisseria*, *Branhamella*, *Acinetobacter*, *Haemophilus* and *Kurthia* constituted a minor proportion of the isolates.

A chi-square test on the frequency of occurrence of Micrococcaceae, Streptococcaeae, Enterobacteriaceae, Bacillaceae and *Corynebacterium* did not reveal any significant difference. A student *t* test on the means of the total bacterial count in the five major groups revealed that organisms of the family Enterobacteriaceae and the genus *Corynebacterium* occurred in significantly higher numbers in the repeat breeder group.

Mycotic flora were detected in 20% of the 142 samples. In the normal fertile group, six of the animals were positive for *Candida* and isolates resembling *Candida*, as against 12 of the cows from the repeat breeder group. Filamentous fungi were isolated from six cows of the normal fertile group and six of the repeat breeder group. The majority of the isolates from both groups were identified as *Penicillium* species along with species of *Aspergillus*, *Alternaria* and *Cladosporium*.

Thirty (21.1%) of the 142 samples yielded

T-strain mycoplasmas and 12 (8.5%) yielded large colony mycoplasmas. All except one of the large colony mycoplasmas were identified as *M. bovis*. The remaining isolate resembled *Acholeplasma laidlawii*. Four of the normal fertile animals yielded large colony mycoplasmas and 11 T-strain mycoplasmas. In the repeat breeder group, eight yielded large colony mycoplasmas and 19 T-strain mycoplasmas.

Although these microorganisms were demonstrated in the cervico-vaginal mucus of both the normal and repeat breeder cows sampled, their significance in the repeat breeder syndrome remains undetermined.

RÉSUMÉ

Cette étude a permis d'obtenir un relevé des bactéries, des champignons et des mycoplasmes du mucus cervico-vaginal, chez 72 vaches normales et fécondes, ainsi que chez 70 vaches infécondes qui manifestaient des problèmes de conception après trois saillies ou plus (*repeat breeders*).

Des études qualitatives permirent d'identifier 25 espèces de bactéries, à partir du mucus cervico-vaginal des vaches de ces deux groupes. Les espèces isolées le plus souvent appartenaient respectivement aux genres suivants: *Streptococcus*, *Micrococcus*, *Staphylococcus*, *Escherichia*, *Bacillus* et *Corynebacterium*. Les espèces isolées moins fréquemment appartenaient par ailleurs aux genres *Proteus*, *Klebsiella*, *Pasteurella*, *Neisseria*, *Branhamella*, *Acinetobacter*, *Haemophilus* et *Kurthia*.

Un test chi-carré sur la fréquence de la présence des Micrococcaceae, Streptococcaeae, Enterobacteriaceae, Bacillaceae et *Corynebacterium* ne révéla aucune différence appréciable. Un test-*t* étudiant sur la moyenne du nombre total de bactéries des cinq principaux groupes démontra que les micro-organismes de la famille des Enterobacteriaceae et du genre *Corynebacterium* se retrouvaient en nombre sensiblement plus élevé chez les vaches infécondes.

On décéla des champignons dans 20% des 142 échantillons. Six des vaches normales et fécondes recelaient *Candida* ou des souches qui lui ressemblaient, comparativement à 12 des vaches infécondes. On isola des champignons filamenteux chez six des vaches normales et fécondes et chez autant de vaches infécondes. L'identification de ces champignons révéla que la plupart étaient des espèces appartenant aux genres *Penicillium*, *Aspergillus*, *Alternaria* et *Cladosporium*.

Des 142 échantillons, 30 ou 21.1% contenaient des mycoplasmes à souche T, tandis que 12 ou 8.5% recelaient des mycoplasmes formant de grosses colonies. Dans tous les cas, sauf un, il s'agissait de *Mycoplasma bovis*. L'autre mycoplasme ressemblait à *Acholeplasma laidlawii*. Quatre des vaches normales et fécon-

des recelaient des mycoplasmes formant de grosses colonies et 11 autres recelaient des mycoplasmes à souche T. Huit des vaches infécondes recelaient des mycoplasmes formant de grosses colonies et 19 autres recelaient des mycoplasmes à souche T.

En dépit de la présence de tous ces micro-organismes dans le mucus cervico-vaginal des vaches fécondes et infécondes, la signification de leur présence chez les dernières demeure indéterminée.

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