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A STUDY OF BACTERIA IN BOVINE SEMEN AND THEIR EFFECT UPON LIVABILITY OF SPERMATAZOA

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SUMMARY

1. The organisms isolated from the semen of 36 dairy bulls used in artificial breeding work were (listed in order of predominance) *Bacilli, Micrococci, Coliform, Hemolytic Streptococci, Non-hemolytic Streptococci, Pseudomonas, Actinomyces, Proteus* and *Yeasts.*
2. Fresh semen having a high hemolytic bacterial count showed a substantial decrease in the livability of spermatozoa under storage conditions as compared to samples having a low hemolytic bacterial count.
3. The addition of hemolytic bacteria to fresh diluted semen caused a substantial reduction of storage time.
4. The addition of micrococci, yeast and non-hemolytic streptococci to fresh diluted semen caused a substantial increase in the storage time, while all other types of organisms caused a definite decrease in the storage time.
5. There was no apparent relationship between the fertility of the sires, livability of spermatozoa, and the total plate count of the semen.
6. Five sires from which *Pseudomonas pyocyaneus* organisms were isolated were low in fertility and eventually all five became sterile.

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INTRODUCTION

While the importance of bacteriological control in the field of artificial insemination has been somewhat controversial in the past, practically all workers feel that more information on this subject is essential. Salisbury, Willett and Gunsalus[15]* called attention to the need for more information concerning the possibility of infections of the female genital tract as related to types of bacteria present in both fresh and stored semen.

It has been recognized for many years that semen is an excellent medium for bacterial growth. Several workers have reported putrefaction of semen when stored at room temperatures and have attributed loss of fertility to this condition. Hammond[7], however, found the incidence of disease in the genital tract of female rabbits no higher when inseminated with semen of high bacterial count than when they were inseminated with semen of a low count controlled by low storage temperature.

One of the most serious problems encountered in the application of artificial insemination is the prolongation of the life of the spermatozoa during storage. Semen must be transported long distances and yet remain in a viable state from several hours to a few days before it is used.

Much information is available as to the effect of such factors as diluents, storage temperatures and pH. Very little is known regarding the effects of the many different types of bacteria encountered in the non-diluted and diluted semen. The effect of the various types of bacteria upon non-living materials has been well established. A much clearer picture is needed to determine the effects of these various bacteria upon living sperm. Due to the inherent sensitivity of the sperm to heat, chemicals and disinfectants in general, the elimination of all bacteria without injury to the sperm is impossible.

In a study of the genital tract of bulls inspected shortly after slaughter, Gillman[5] reported finding *Pseudomonas pyocyaneus* along with unidentified rods, micrococci, streptococci and coliform organisms. His findings agreed with those of Williams and Kingsbury[19] as to normal bulls being relatively free of bacteria, whereas impotent bulls harbored large numbers of bacteria.

Williams and Kingsbury[19] reported finding micrococci, hemolytic and non-hemolytic streptococci, coliform and *Brucella abortus* in semen recovered from

*Superscript numerals refer to Bibliography on page 11.

the vagina of cows immediately after service. The sheaths and vaginas had been douched and disinfected previous to service. Webster[18], using the same collecting technique, isolated and identified micrococci and diptheroids from normal semen. On the other hand, semen from bulls in areas of enzootic sterility contained, in addition, alpha hemolytic streptococci. This method of collection has been criticized as increasing risk of vaginal contamination.

In work done by Hatziolis[8] and cited by Gunsalus, Salisbury and Willett[6], it was found that almost every ejaculate yielded bacteria consisting of proteus, coliform, cocci, pseudomonas and spore forming rods. The semen was collected aseptically by the use of the artificial vagina.

Gunsalus, Salisbury and Willett[6] recorded plate counts of semen from bulls with uncleaned sheaths from 10 to 100 fold higher than those which previously had been cleaned. They found pseudomonas, coliform, diptheroids, bacilli, staphylococci, and, in one case, hemolytic diptheroids present in the semen. A high percentage of diminishing fertility in bulls within six months after the isolation of *Pseudomonas pyocyaneus* from the semen was reported. In this same study, it was concluded that there was no appreciable germicidal action of semen and that living cultures of *E. coli* added to the sperm gave marked superior motility rating over the untreated controls.

The control of certain bacteria by the use of bacteriostatic compounds is advocated and recommended by many investigators. Shettles[16] observed that the survival and activity of human spermatozoa was not reduced by the addition of sulfapyridine or sulfanilamide in concentrations up to and including 160 mg. per 100 ml. of diluent. Knodt and Salisbury[11] working with bull semen, found that the addition of 200 mg. or more sulfanilamide per 100 ml. of yolk-citrate diluter controlled the growth of bacteria in stored, diluted semen for 20 days at 5°C. In this study it is reported that 300 mg. sulfanilamide per 100 ml. of diluter was considered optimum, since it not only prevented bacterial growth but also brought about a significant increase in the livability of the sperm. Later[14], these workers observed that the use of 300 mg. of sulfanilamide per 100 ml. of diluter gave an increase in the fertility of bull semen used routinely in the breeding of dairy cows. They attributed this increased fertility resulting from the addition of sulfanilamide to possible changes in the metabolism of the sperm, rather than to the control of bacteria growth alone.

The effects of penicillin, when added to diluted semen as an antibiotic agent, were reported by Almquist, Thorp and Knodt[1]. They found no significant decrease in the motility of diluted, stored semen when concentrations of penicillin ranging from 0 to 1000 units per ml. were added. However, levels of penicillin ranging from 1000 to 2000 units per ml. brought about a significant decrease in the motility during storage. In this study, it was found that penicillin retarded bacterial growth in all levels for a period of eight days.

Phillips and Spitzer[12] recommended that .03 per cent sulfathalidine, sul-

fasuxidine or streptomycin be added to their LGB (lipid, glucose, buffergum) diluter for the control of bacterial contamination. They also mentioned that penicillin, used in proper amounts, was not toxic to the sperm.

Salisbury[14] reported that the addition of glucose to sperm in storage increased the motility and storage time. He also observed that the addition of sulfanilamide promoted the livability of the sperm but depressed the glucose utilization to amounts less than the lactic acid produced.

METHODS AND MATERIALS

The semen used in the study of total and differential plate counts was obtained from bulls at the M. F. A. Breeding Farms at Springfield, Missouri. All other studies, including those on bacterial types and their subsequent effect on sperm, were made on semen collected from bulls in the University herd. In both herds the handling of bulls was essentially the same. The bulls were confined to stalls, except for exercising and collection, and were not used for natural breeding. They were brushed around the underline and sheath prior to collection.

The semen was collected with artificial vagina (Herman and Ragsdale[9]) which had been previously sterilized by autoclaving all glass parts and rinsing the rubber parts with 70% alcohol. Immediately after collection, the semen was diluted and cooled. The rates of dilution varied from 1 : 10 to 1 : 15 depending upon the amount of semen available. Yolk citrate, (Salisbury, Fuller and Willett[13]) and pabulum (Phillips and Spitzer[12]) diluters were used. These diluents were prepared as aseptically as possible without autoclaving and plate counts revealed good bacteriological control.

The organisms were isolated from fresh semen by the streak and poured plate techniques. Both blood agar (containing 2% defibrinated cow's blood) and nutrient agar were used for the isolation and determination of total and differential plate counts. All plates were incubated at 37°C. for three days. This incubated temperature was considered optimum for the growth of organisms originating in the bull. Standard laboratory media and techniques were employed for the identification of the organisms[2, 3, 10, 20].

Cultures for the inoculation of diluted semen for storage tests were maintained in a peptone #3 broth[3]. Care was taken to have the cultures in a viable, active condition at the time of addition.

For the extraction of the bacterial filtrate, sintered glass Buechner funnels of a filter grade of "G 5 on 3" were used. The filtrates were then checked for purity by the streak plate method.

In general, the determination of the effects of bacteria on sperm motility during storage were made in the following manner: The semen was collected as described above. The warm semen was diluted, immediately upon arrival at the laboratory, with a diluter of the same temperature. After dilution, it was added to sterile storage tubes at the rate of 4.5 ml. per tube. Using aseptic

technique, the cultures or culture filtrates as the experiment called for, were added at the rate of 0.5 ml. per tube of diluted semen. Semen control samples contained 4.5 ml. of diluted semen and 0.5 ml. of plain peptone #3 broth. After addition, the tubes were rotated gently to facilitate mixing and stored at 5°C. until expiration of motility.

Motility ratings, using the technique proposed by Swanson and Herman[17], were run upon the original semen, the fresh diluted semen, and every twenty-four hours thereafter on the inoculated diluted semen and controls while in storage at 38 to 40°F.

EXPERIMENTAL DATA AND RESULTS

Types of Bacteria Present in Fresh Semen. The types of bacteria found in the ejaculates of 36 bulls that had good conception rates were as follows: (listed in order of predominance) Bacilli, Micrococci, Coliform, Hemolytic Streptococci, Non-hemolytic Streptococci, Pseudomonas, Actinomyces and Proteus. Yeasts were also isolated. Of these all have been previously reported with the exception of Actinomyces and Yeast. These occurred 13 and 3 per cent, respectively, in all the ejaculates studied. Of the 36 bulls analyzed, 5 were found to have infections of *Pseudomonas pyocyaneus*, later causing sterility in 2 bulls and very low fertility in the other 3. At the time of writing, all of these bulls have been disposed of because of sterility.

The semen of three sterile bulls was also examined during this study. In each case, the bacterial flora was essentially the same, consisting predominantly of coliform, bacilli and cocci. No hemolytic organisms were found.

Relation of Total and Hemolytic Bacterial Counts of Fresh Semen With the Length of Storage Time. This study was undertaken in an attempt to determine whether any relation actually exists between the different bacterial numbers in fresh semen and its subsequent livability during storage. Included in this work were 20 bulls, with an examination of 64 ejaculates. Estimates of bacterial numbers were made by the plate count method. Both 2% defribrinated blood agar and nutrient agar were used, but the results from the nutrient agar were so questionable that they could not be accurately interpreted.

The data in Table 1 indicate the relation of the total and hemolytic plate counts of fresh undiluted semen with storage time of the diluted semen. Of the 20 bulls studied 10 produced semen with a total plate count of 50,000 bacteria per cc. or more, while the other 10 had total counts under 50,000 bacteria per cc. The semen with the low total count stored an average of 0.8 days longer than that having the high count. This increase, however, was not considered significant. On the other hand, semen containing 1,000 hemolytic bacteria per cc. or under stored, on the average, 2.2 days longer than that having a plate count over 1,000 hemolytic bacteria per cc.

Since the number of samples in the foregoing experiment was small, the study was continued using fresh diluted semen secured from the M. F. A.

TABLE 1--RELATION OF TOTAL AND HEMOLYTIC PLATE COUNTS OF FRESH UNDILUTED SEMEN WITH SUBSEQUENT STORAGE TIME OF THE DILUTED SEMEN

Bull	Plate Counts (64 ejaculates)		Motility Ratings					Days before expiration of motility*
			Original undiluted semen	Diluted semen stored at 5° C.				
	Total	Hemolytic		24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	
1	192,000	Too numerous to count	4+	3	2+	1	0	3
2	6,400	1,000	4	3	2+	2+	2	7
3	9,200	1,400	4+	3	2+	2	1+	4
4	6,400	300	4	3-	2	2	1	4
5	900	100	4	3	2	2	1	4
6	272,000	1,000	4+	3	2+	2+	2+	7
7	691,000	102,400	4+	3	2	2	1+	4
8	7,400	700	4	3	2	2-	2-	5
9	160,000	32,000	4	3	2+	2	2-	5
10	23,700	700	5	3	3	2+	2+	8
11	236,800	44,000	4+	2+	2	1	0	3
12	450,200	6,200	4	3	2	1	0	3
13	30,000	2,500	4	2+	1	0	0	2
14	6,700	900	3+	3-	2+	2+	2	5
15	162,500	Too numerous to count	4+	2	1	0	0	2
16	2,304,000	Too numerous to count	4	2	2	2	1	4
17	128,000	4,900	4+	3	2	2	2	5
18	96,000	>100	4+	3	3-	2+	2+	5
19	3,000	>100	4	3	2+	2+	2	6
20	1,100	>100	4+	3	2+	2+	2+	6

* Motility rating of "1" regarded as expired.

Breeding Farms at Springfield, Missouri. The data from 40 bulls which included 74 additional ejaculates are included in Tables 2 and 3.

Table 2 indicates the relationship of total numbers of bacteria in the fresh diluted semen to its subsequent storage time. Dividing the samples into two parts, regarding 16,000 bacteria per cc. or less as low count and more than 16,000 bacteria per cc. as high count, it was found that the samples in the high count group stored an average of 6 hours longer than those with the low counts. The reason that a lower standard for high and low counts in this study as compared to the earlier work was due to the different season of the year. The data in Table 1 was compiled during the summer months while the data in Table 2 were derived during the fall, winter and early spring. This

TABLE 2--RELATION OF TOTAL PLATE COUNTS OF DILUTED SEMEN AND SUBSEQUENT STORAGE TIME

Total bacteria per cc. of diluted semen 8 hours old	A study of 74 (40 bulls) samples stored at 5°C.							
	Days in storage before expiration of motility*							
	1	2	3	4	5	6	7	8
1,000		2	3	4	2	4		
1,000 - 4,000			1	4	4	4	1	1
4,000 - 8,000		3		3	1	2	1	
8,000 - 12,000			1	1	1			
12,000 - 16,000				2	1	2		
16,000 - 20,000			2		2			
20,000 - 24,000	1		1	1		1	1	
24,000 - 30,000				1	1	1		
30,000		2	1	1	3	5	1	1

*Motility ratings of "1" regarded as expired.

accounts for most of the lower counts found in Table 2 since the temperature conditions are below optimum for growth of the bacteria previous to the storage of the semen.

In the study of hemolytic bacteria, it was again found that high hemolytic bacterial counts were detrimental to the storage of diluted semen. The data in Table 3 were also divided into two groups. Samples with 1,000 hemolytic bacteria per cc. or less were regarded as low count semen. It was found that the samples having low plate counts stored on the average of 30 hours longer than those having a high count.

TABLE 3--RELATION OF HEMOLYTIC PLATE COUNTS OF DILUTED SEMEN AND SUBSEQUENT STORAGE TIME

A study of 74 (40 bulls) samples stored at 5°C.								
Hemolytic bacteria per cc. of diluted semen 8 hours old	Days in storage before expiration of motility*							
	1	2	3	4	5	6	7	8
>100		2	5	5	4	11	2	2
100 - 300				3	6	3		
300 - 500				3	1	1	1	
500 - 700				1				
700 - 900								
900 - 1100								
1100 - 1300								
1300 - 1500					1			
1500 - 1700						1		
1700 - 1900		1	1	1	1			
1900 - 2100			1	1		1		
<2100	1	4	2	3	2	2	2	1

* Motility ratings of "1" regarded as expired.

Growth of Bacteria in Diluted Semen During Shipment. Since semen and diluters provide such an excellent medium for bacterial reproduction, a study of their growth during shipment was undertaken. A diluted sample from each of 19 ejaculates was plated at the breeding farm. The samples were then shipped to the University laboratory where they were again plated. The time required for shipment of the semen from the breeding farms to the laboratory was 8 to 9 hours. Temperatures, during shipment, were controlled by the use of insulated cans. The average temperature upon arrival of the samples was 7°C.

Table 4 shows a comparison between the numbers of bacteria in fresh diluted semen and the same diluted semen 10 hours later. Both total and hemolytic counts are included. Samples 12 and 14, for no apparent reason, showed a greatly reduced hemolytic count during shipment. Since all samples were treated in the same manner, no reason was seen for excluding these samples from the data.

Effect of Bacterial Inoculum Upon Motility of Diluted Semen During Storage. This study was undertaken to determine the effect of hemolytic bac-

TABLE 4--BACTERIAL GROWTH IN DILUTED SEMEN DURING SHIPMENT AT 7° C.

Bacteria per cc. of diluted semen				
	Plate Counts of Fresh Diluted Semen		Plate Counts of Diluted Semen 10 Hours Old	
Bull	Total	Hemolytic	Total	Hemolytic
1	5,500	Too numerous to count	5,100	Too numerous to count
2	1,800	150	54,800	5,700
3	320	60	1,100	750
4	300	35	11,000	560
5	330	25	1,200	600
6	100	5	340	240
7	250	12	1,400	900
8	30	3	4,900	390
9	9,600	75	32,200	9,900
10	480	3	6,800	110
11	9,100	35	7,900	500
12	27,650	4,000	52,000	40
13	250	25	37,100	470
14	8,000	1,600	5,700	60
15	700	160	78,300	290
16	1,580	110	5,500	340
17	9,500	1,760	12,100	1,600
18	15,000	260	37,500	2,000
19	2,000	140	5,400	130

teria upon motility of sperm during storage. However, when preliminary experiments revealed an increase in motility upon addition of certain non-hemolytic bacteria, the study was expanded to include all types of bacteria previously isolated from semen.

In this study, various bacterial cultures were added to a total of 257 samples of fresh diluted semen for storability studies. The effects of the 10 different organisms were observed on these samples, resulting from the dilution of 27 ejaculates from 6 bulls. Controls were run with each series of bacteria studied. Both yolk citrate and pabulum diluters were used.

It was surprising to note that bacteria did increase the longevity of the sperm, Table 5. Micrococci, yeast and non-hemolytic streptococci resulted in an average increase of 47, 22 and 18 hours, respectively, in the storage time of semen.

All other bacteria used in this study brought about some decrease in the livability of the sperm. This difference was especially noticeable in semen samples inoculated with cultures of Actinomyces and hemolytic Bacilli. The effect of *Pseudomonas pyocyaneus* was not as detrimental to the sperm as was anticipated.

An attempt was made to determine whether the detrimental or beneficial result, as the case may have been, of the bacteria on sperm motility could be brought about without the living culture. The heated culture, filtrate and heated filtrates, from beneficial bacteria had no significant effect on the motility of

TABLE 5--THE COMPARISON OF THE LENGTH OF STORAGE OF INNOCULATED DILUTED SEMEN SAMPLES AND THEIR RESPECTIVE CONTROLS

(6 Bulls - 4 or More Ejaculates Studied for Each Bull)

Organisms	Storage Time in Hours						Average Storage Time For All Bulls	Number of Hours Samples Stored Longer Than Control	Number of Hours Samples Stored Less Than Control
	Bull								
	A	B	C	D	E	F			
Controls	96	72	96	72	96	120	92		
Bacilli Alpha Hemolysis	60	24	48	48	72	72	54		38
Bacilli Beta Hemolysis	64	48	48	72	72	96	67		25
Micrococci	132	120	144	126	144	168	139	47	
Coliform	72	36	72	72	96	96	74		18
Streptococci Alpha Hemolysis	84	48	72	72	48	102	71		21
Streptococci Non-Hemolytic	120	84	108	96	96	156	110	18	
Pseudomonas Pyocyaneus	96	72	84	48	72	96	78		14
Actinomyces	48	60	48	36	48	84	54		38
Proteus	48	72	72	48	84	72	66		26
Yeasts	120	96	108	108	96	156	114	22	

the sperm during storage. Cultures of a detrimental nature treated in the same manner showed no signicant variations in the sperm motility, although a slight decrease was noted for some of the organisms.

DISCUSSION

In the past many studies have been made to determine the bacterial flora of fresh semen. Although many variables exist, such as technique of collection, preparation of the bull and possible contamination in general, the normal flora has been reasonably well established. All bacterial types previously reported were found in this study with the exception of staphylococci, diptheroids and Brucella. However, two additional organisms, *actinomyces* and *yeast*, were found in limited cases.

In the study of total bacterial count, no relation could be found to exist between total bacterial count and storage time. Since different bacteria have varied effects upon the stored semen, these findings are not surprising. However, the detrimental effects of high numbers of hemolytic bacteria in fresh semen in relation to storage time was clearly demonstrated. The origin and function of these hemolytic bacteria is not clearly understood. In the case of *Pseudomonas pyocyaneus*, a clearly pathogenic form, the detrimental effect on motility seems to originate within the bull and to exhibit a physiological effect upon the production of semen rather than upon the semen itself. Bulls heavily infected with this organism seem to undergo a gradual decrease in spermatozoa motility ratings, and finally sterility results thus indicating that a physiological effect upon the production of the semen is brought about.

Growth of bacteria in semen during shipment at 7°C. was shown to occur at a rather slow rate. This fact was also shown by Gunsalus, Salisbury and Willett[6] using a storage temperature of 5°C. It should again be mentioned

that there was a sharp decrease in the hemolytic counts of certain samples during shipment for no apparent reason.

The addition of hemolytic bacteria to diluted semen brought about a pronounced decrease in livability of the sperm during storage. In view of the detrimental effects of high hemolytic counts on motility of semen during storage, this was anticipated. However, it was surprising to note that the additions of micrococci, yeasts and non-hemolytic streptococci brought about a definite increase in storage time of the diluted semen.

The question immediately arises as to what agents from these bacteria are responsible for the decrease or increase in the storage time of the sperm. In the case of hemolytic bacteria, it is logical to assume that the detrimental effects are brought about by products toxic to the sperm. As no immediate increase in motility was observed, the theory of stimulation of the sperm causing a shortening of the storage period was ruled out. Jordan and Burroughs[10] reported that spermatozoa inoculated into the blood stream of a living animal was first rendered motionless and later lysed by the complement of the blood. Since hemolytic bacteria do possess lytic properties, it is possible that these factors are responsible in part at least for the detrimental effects brought about.

As the bacterial filtrate studies have shown, the living beneficial bacteria must be present to increase the storage time of the diluted semen. Therefore, it would seem that either the immediate by-products of the living bacteria or the bacterial utilization of harmful sperm by-products cause the beneficial results. Bacterial production of enzymes, which are either beneficial in sperm metabolism or act on harmful sperm waste products, is another likely explanation.

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