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A COMPARISON OF THE CALIFORNIA MASTITIS
TEST WITH THE OTHER COMMONLY EMPLOYED
DIAGNOSTIC TESTS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Jose Britto Figueiredo
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A COMPARISON OF THE CALIFORNIA MASTITIS TEST
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DIAGNOSTIC TESTS

by

Jose Britto Figueiredo

AN ABSTRACT


Submitted to the College of Veterinary Medicine
Michigan State University of Agriculture and
Applied Science in partial fulfillment of
the requirements for the degree of

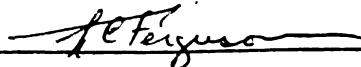
MASTER OF SCIENCE

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Approved





The California Mastitis Test (CMT), a new indirect test for detection of bovine mastitis, was applied in 70 milk samples and its value was compared with seven commonly employed diagnostic tests.

All samples were from mid-lactation phase and from chronic or sub-clinical mastitis.

The CMT is closely correlated with the Whiteside test, but instead of sodium hydroxide, a surface active agent and bromcresol purple are used as reagent.

The per cent agreements among the methods of diagnosis used and bacteriological isolation and identification (considered standard method) were: pH test 19.6; leucocyte counts 35.1; catalase test 41.2; Hotis test 46.6; Whiteside test 47.5; CMT 48.8 and bacterioscopic examination 54.7.

Bacteriological isolation and identification were made on Edwards' medium and on the Tellurite-Glycine Agar medium described by Zebovitz, Evans and Niven (1955), which is a modification of Ludlam's medium. This medium is very efficient to isolate coagulase producing strains of micrococci and showed a per cent agreement of 83.0 as compared with the test of coagulation of blood plasma.

The literature of the micrococci pathogenicity, evaluated by their ability in producing cogulase and hemolysins, was reviewed and discussed. The total agreement between coagulase and hemolysins production occurred only with the alpha-beta hemolytic Micrococcus pyogenes.

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This thesis is dedicated
to my PARENTS and to my WIFE
who have been the inspiration of
my search for greater knowledge.



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CHAPTER I

INTRODUCTION

If there is a subject in the entire field of animal pathology to which basic research is always needed it is mastitis or, more specifically, bovine mastitis.

Thousands of papers have been written and still many points remain obscure or not well defined with respect to pathology, etiology, diagnosis, treatment and, of course, control.

The subject is really complex and, unfortunately, the foremost research workers in this field very often have different concepts. This situation led Murphy (1956) to write, an excellent review of the whole picture, fixing some important points and suggesting a general understanding.

Outbreaks of milk-borne human diseases have declined due to extensive milk pasteurization and several types of bacteriological and serological diagnostic methods, but bovine mastitis continues as a very important dairy herd infection.

The significance of carriers is great in almost all animal infections, particularly in diseases like bovine mastitis where no conclusive evidence has been found for an artificial or even natural lasting immunity. Thus a rapid

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and precise diagnostic method is desired for the efficient treatment and establishment of a practical control program.

With this objective in mind research workers at the University of California recently developed a new test for the detection of abnormal milk, which is called California Mastitis Test (CMT).

The purpose of this study, which has no pretension to fill deficiencies or vacuums, is to compare the new CMT with 7 currently employed laboratory methods of diagnosis for mastitis along with the medium described by Zebovitz, Evans, and Niven (1955) for the isolation of coagulase positive micrococci.

CHAPTER II

MATERIAL

Samples were collected from five different farms located in four counties in Michigan.

The udders were washed with warm aqueous chlorine solution containing 200-400 parts per million of available chlorine. Special care was taken on the end of the teat which was re-washed in order to get the teat meatus as bacterial free as possible.

The fore-milk was used to perform the CMT, and subsequent streams were used to carry out other tests.

Samples were always collected from each quarter just before milking, however, composite samples were also taken from known negative cows.

All milking cows from which samples were collected were in the mid-lactation phase in order to avoid expected alterations in the milk, since leucocyte counts are known to be higher in the early and the late lactation.

In this thesis were included only chronic mastitis or sub-clinical infections, which are latent and hence auxiliary methods of diagnosis must be applied.

Seventy milk samples were obtained from normal and abnormal udders.

CHAPTER III

METHODS

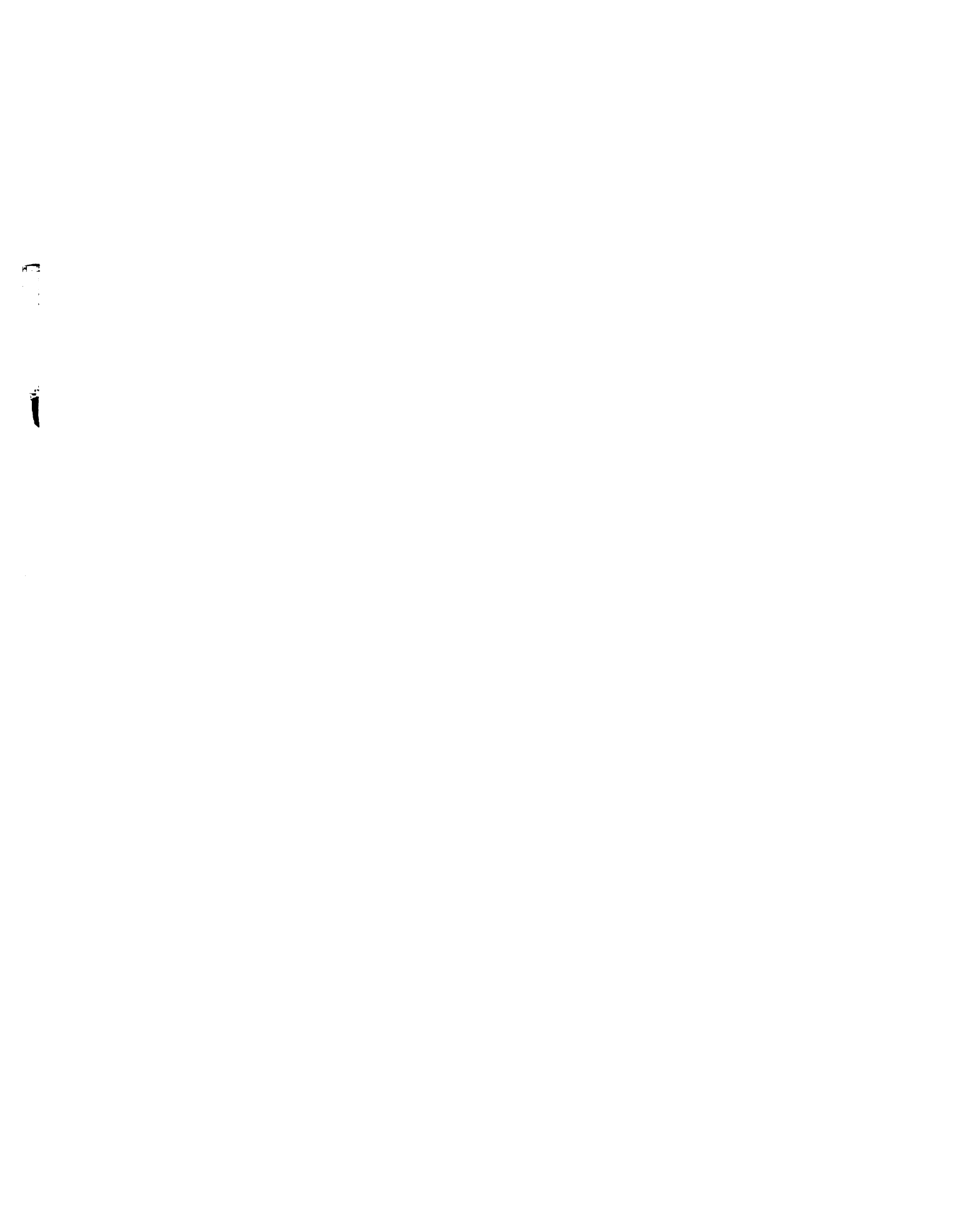
Generally standard or well accepted techniques were utilized whenever available.

Leucocytes Counting

The technique of Prescott and Breed (1910) was used with the following modification. Instead of measuring the milk by means of a graduated pipette, a closed platinum loop which delivered a known amount was used for spreading the milk on the guide area. This procedure is authorized by the Standard Methods for the Examinations of Dairy Products (1948). According to Malcolm and Smillie, cited by Blackburn, Laing, and Malcolm (1955), there is no significant variation between the two procedures.

Samples were examined after incubation. No significant variations occur in cell counts if the film is made before or after incubation. This was suggested by Bryan (1935), and established by Slanetz and Naghski (1939).

The staining method employed was that suggested by Charlett (1954) which is similar to that of Broadhorst-Paley but does not require previous fixation and is performed in 10-12 seconds. The differential coloration makes leucocyte counting easier and more accurate. Cells in 25 fields were counted to determine the number per ml. of milk.



Modified Whiteside Test

This test described by Whiteside (1939) was performed in accordance with the modification of Murphy and Hanson (1941) and Murphy (1942). Two drops of normal sodium hydroxide and 5 drops of fresh milk, or 1 drop of normal sodium hydroxide to 5 drops of refrigerated milk were mixed on the surface of a plate commonly employed for the Brucella agglutination test.

Readings were taken 20 seconds after mixing with a toothpick. The reactions were scored according to the directions given in that paper.

Catalase

The method used was suggested by Merchant and Packer (1945) in which 5 ml. of 1 per cent hydrogen peroxide were added to 15 ml. of fresh milk in a Smith fermentation tube. Positive reactions were those in which over 1.5 ml. of gas was accumulated in the blind arm of the tube after incubation at 37°C. for 3 hours.

California Mastitis Test (CMT)

The technique of Schalm and Noorlander (1957) was employed. Two or 3 ml. of milk were drawn into a special plastic paddle from each quarter of udder. A similar quantity of reagent was added by using a polyethylene wash bottle. Positive reactions appeared immediately after the mixing of reagent and the milk. During the period of observation,



the paddle was maintained in a gentle rotating motion. Reactions were scored in the prescribed manner, that is, based on the presence and intensity of precipitate or gel formation. However, a classification designated as 4 was utilized when an udder secretion was abnormal to the eye which is commonly designated as garget. Also, pH was indicated by changes in the color of the indicator.

Schalm (1957) explained the chemical basis of the CMT in the following statement: "Chemical compounds belonging to the group of surface active agents containing long-chain hydrocarbon salts have been found to become visibly altered in the presence of native proteins of cellular origin." The surface-active agent used is anionic and bromcresol purple is added as an pH indicator.

Hydrogen-ion Concentration

The electrometric method employing a Beckman Glass Electrode pH Meter, model H 2, was used.

Bacterioscopy

A thin smear was prepared and stained with the Charlett (1954) improved staining method after an incubation period of 12-20 hours at 37°C., as suggested by Bryan (1935, 1941) and Little and Plastridge (1946).

Hotis Test

This method was proposed by Hotis and Miller (1936) for the detection of mastitis due to streptococci. Further

observations permitted it to be used for the other mastitis organisms. To carry out this test, 9.5 ml. of fresh or refrigerated milk were added to 0.5 ml. of a sterile aqueous bromocresol purple solution.

Two readings were made: one at 24 hours and another at 48 hours of incubation at 37°C.

Bacteriological Isolation and Identification

Two loops (4 mm. diameter) of fresh or refrigerated milk were plated on Edwards' medium (1933) and on the Tellurite-Glycine Agar medium¹ described by Zebovitz, Evans and Niven (1955), and then incubated at 37°C. This medium is a modification of Ludlam's medium and as such, has potassium tellurite and lithium chloride as selective agents. Glycine is added to increase selection. The final pH of the medium is adjusted to 7.2. By the use of these two media, cocci and bacillar organisms (Pseudomonas sp., E. coli and Proteus sp.) can be easily isolated, with the advantage that coagulase positive Micrococcus pyogenes is identified in 24 hours by its black colonies on the Tellurite-Glycine Agar medium. Very few other micro-organisms can grow on this medium. Micrococci which are coagulase negative are recognized because they grow in grey colonies.

¹Supplied by Case Laboratory, Chicago, Illinois.

To confirm the ability of the black colony in coagulating the plasma, it was transferred to a Brain Heart Infusion and, after 16-24 hours of incubation at 37°C., 2 drops were used as inoculum to 0.5 ml. of plasma solution. Bacto-coagulase plasma,² which is desiccated rabbit plasma, was used. Boyd (1956) compares the use of desiccated plasma with fresh citrated plasma and reported identical results.

The presence of catalase, gelatinase and hemolysin production on 5 per cent bovine blood agar were also checked in all cultures of cocci. Also, the capacity to hydrolyze sodium hippurate and metabolize some carbohydrates like mannitol, esculin and insulin were tested.

Brilliant-Green-Bile 2 per cent broth, gelatin, litmus milk, pigment production and a few sugars were used to isolate and identify Pseudomonas sp. and Escherichia coli.

The Gram's staining characteristics were checked for all organisms isolated.

²Supplied by Difco Laboratories, Inc., Detroit, Michigan.

CHAPTER IV

RESULTS

A criterion to compare efficiency of the CMT and other currently used methods of diagnosis of mastitis was formulated based primarily on the isolation and identification of the etiological agent.

A complete identification of each etiological agent was not made, because it was judged unnecessary for the objective of this work. The tests carried out were considered sufficient to permit grouping them in the following categories of infections: (1) due to Streptococcus agalactiae, (2) due to other streptococci, (3) due to Micrococcus pyogenes, and (4) infections due to Pseudomonas sp. or Escherichia coli. These categories were stated by Murphy (1956) as accounting "for at least 99 per cent of all mastitis."

Isolation and Identification

In this study, a total of 54 micrococci were isolated from 70 milk samples. Of these, 29 were coagulase positive and 25 were coagulase negative strains.

The selective medium employed to isolate coagulase positive strains, gave typical black colonies (coagulase producers) in 32 instances, and grey colonies (coagulase



negative) in 29 instances. Twenty-nine of the 32 black colonies were confirmed as coagulase positive and all the grey colonies were coagulase negative. Thus, false positive results were found 3 times. The per cent agreement of these results are given in Table I.

TABLE I
EFFICIENCY OF THE TELLURITE-GLYCINE AGAR MEDIUM FOR
THE ISOLATION OF COAGULASE PRODUCING MICROCOCCI

Compared Medium	Coagulase Producers								Per Cent Agree- ment
	Positive Findings				Negative Findings				
	Positive No.	%	Negative No.	%	Positive No.	%	Negative No.	%	
Tellurite- Glycine Agar	29	100.	0	0.0	3	12.0	22	88.0	83.0

Gelatinase production--Of the coagulase positive micro-organisms, 81.4 per cent were gelatinase negative, whereas only 18.6 per cent were gelatinase positive.

Hemotoxins--Of the total micrococci identified, 36 strains were hemotoxigenic as follows: 16 alpha-beta; 17 beta and 3 alpha. The 29 coagulase producing strains were: 13 beta hemolytic and 16 alpha-beta hemolytic. The 25 coagulase negative were 4 beta hemolytic, 3 alpha hemolytic and 18 non-hemolytic. The total agreement between coagulase positive and hemolysin production occurred only with the alpha-beta hemolytic Micrococcus pyogenes.

Catalase--All isolated micrococci, coagulase negative or coagulase positive, were catalase positive whereas the streptococci were catalase negative.

From 70 milk samples, 21 streptococci were isolated. Based on the results obtained from the tests used, the microorganisms were thus grouped: 4 Streptococcus agalactiae; 17 other streptococci (3 were Streptococcus uberis and 4 Streptococcus dysgalactiae). Mixed infections were found in 7 instances with Micrococcus pyogenes, and in 3 samples with Pseudomonas sp. or E. coli.

With hemolysin production, the findings were: 9 alpha (virescent), 4 beta and 8 gamma (non-hemolytic).

Bacillary infections were found 11 times due to Pseudomonas sp. or E. coli. The latter was isolated in 10 instances and the first in one instance. Mixed infections were found 3 times with streptococci and 5 times with micrococci.

Of the 70 milk samples collected, 51 were bacteriologically classified as positive and 19 as negative.

Leucocyte Counts

In this study, 29 samples were found to be positive (leucocyte counts above 500,000 per ml.) and 41 negative. False positive were found 3 times. The 41 leucocyte counts (58.5 per cent) that were found negative, contained less than 500,000 per ml.; 11 samples (15.7 per cent) contained

500,00 to 1,000,000 per ml., and finally 18 samples (25.7 per cent) had 1,000,000 or more leucocytes per ml.

The results refer to the total cells. Differential and total counts have no market advantage, except in milk from late lactation. (Blackburn, Laing and Malcolm--1955).

Hydrogen-ion Concentration

The characteristics of milk used in this study made it possible to regard those which had a pH value of 6.9 or more as positive samples.

Ten samples were positive (14.3 per cent) and 60 were negative (85.7 per cent). Forty-one negative samples came from mastitic milk recognized by bacteriological findings. This means that 80.4 per cent were false negative results.

Whiteside Test

The results were: 50 positive samples (71.5 per cent) and 20 negative samples (28.5 per cent). False positives were obtained 7 times (14.00 per cent) and false negative 8 times (40.0 per cent).

Catalase

Thirty samples (42.8 per cent) were classed as positive and 40 as negative (57.2 per cent). False negative results were found in 23 samples (57.2 per cent).

Bacterioscopy

The results refer only to cocci-streptococci or micrococci. Bacteriologically, 43 samples (61.4 per cent)

were found to contain pathogenic cocci, in pure or mixed infections, and 27 non-pathogenic cocci. Size and morphologic characteristics were the criteria for classification. The bacterioscopic findings were positive in 52 instances and negative in 18 instances. The distribution of false results are given on Table II.

TABLE II

EFFICACY, IN TERMS OF PER CENT AGREEMENT, OF THE BOVINE MASTITIS TESTS WHICH WERE STUDIED IN COMPARISON TO THE BACTERIOLOGICAL FINDINGS

Compared Tests	Bacteriological								Per Cent Agreement
	Positive Findings				Negative Findings				
	Positive No.	%	Negative No.	%	Positive No.	%	Negative No.	%	
Leucocytes counts	26	50.9	25	49.1	3	15.8	16	84.2	35.1
pH	10	19.6	41	80.4	0	0.0	19	100.0	19.6
Whiteside	43	84.3	8	15.7	7	36.8	12	63.2	47.5
Catalase	28	54.9	23	45.1	2	10.6	17	89.4	41.2
Bacterioscopic*	41	95.4	2	4.6	11	40.7	16	59.3	54.7
Hotis*	36	83.7	7	16.3	10	37.1	17	62.9	46.6
CMT	41	80.4	10	19.6	6	31.6	13	68.4	48.8

*Examination for both cocci groups, only.

Hotis Test

The criterion used to score Hotis reactions due to streptococci was that described by Hotis and Miller (1936) and Miller (1943). To read the reactions supposedly due to Micrococcus, the characteristics described by Miller (1943) and Schalm (1948) were used. This is based mainly on the digestion of the tested milk by the pathogenic micrococci.

In the 70 milk samples the Hotis test was classified as positive 46 times and as negative 24 times. Comparisons with the bacteriological findings are presented in Table II.

California Mastitis Test (CMT)

This test gave 47 positive results and 23 negative results. Some positive and negative results were classified as false and their distributions are found in Table II.

Per Cent Agreement

The comparison of the results from each type of test with the bacteriological findings was made in the form of per cent agreement. This was calculated as follows: the sum of the true results in per cent minus the sum of false results in per cent, all divided by two. Per cent agreement was used to express the relative efficacy of the tests employed. This method gives an idea of the real value of the figures obtained.

There were cases in which the test gave 100 per cent agreement in judging negative samples, such as pH, but showed very low relative efficacy in respect to per cent agreement. A low per cent agreement means, in this case, a high number of false results.

CHAPTER V

DISCUSSION

The standard of comparison used in this thesis was the bacteriological findings. This criterion can be criticized because it is possible to find pathogenic bacteria in the milk but no clinical disease. On the other hand, inability to isolate pathogenic micro-organisms does not mean, necessarily, that the udder is free of infection.

Also, some physical-chemical changes in the milk can persist after the udder is made free of infection. Irritating effects due to injection of some drugs into the udder may be responsible for the increase in the abnormality of resulting secretion. Drury (1952) has demonstrated this in the case of Neomycin sulfate. He states that, "the irritation from the treatment was not as severe as the infection when judged on the basis of the leucocyte counts, chloride content, pH and resazurin class." Since samples were taken from untreated cows or from those treated sometime ago and now with chronic or sub-clinical infection, some of the obstacles were eliminated.

The choice of the methods used for comparison was based on their relationship to the new CMT. Others were employed because they are commonly applied in the routine

control of bovine mastitis. A comparison of some of these tests to cultural examination as reported in the literature is summarized in Table III.

Leucocyte Counts

In normal milk cows, there is variation of cell number from early, to mid and late lactation, also during the season of the year, and even between morning and evening milkings. The borderline of normality is not sharp and has received several interpretations from different workers.

Plastridge, Anderson and Williams (1939) found that 80.2 per cent of 2,125 milk samples free of mastitis organisms, had under 100,000 cells per ml.; 17.7 per cent had between 100,000 to 500,000 cells per ml.; 2.0 per cent had 500,000 to 1,000,000 cells per ml. and only 0.1 per cent contained over 1,000,000 cells per ml.

Recently, Schalm (1957) reported similar results on 690 normal milk samples from the second week to the seventh month of lactation. He found that 76.52 per cent had cell counts under 100,000 per ml.; 19.99 per cent between 100,000 to 500,000 cells per ml.; 2.31 had 500,000 to 1,000,000 per ml. and finally, 1.15 per cent of the samples were classified as over 1,000,000 cells per ml.

McFarlane and Blackburn (1949) in comparing the cell counts of the milk with "post mortem" histological findings in the udder, reported an agreement in 92 per cent of cases. Emphasis must be placed on the fact that those authors have

TABLE III

COMPARISON OF BACTERIOLOGICAL AND OTHER DIANOSTIC METHODS FOR
THE DETECTION OF BOVINE MASTITIS (PER CENT)

Authors	Bacterioscopy		Leucocyte Counts 500T or more		Hotis Test		pH Test		Catalase Test	
	Posi.	False Posi.	Posi.	False Posi.	Posi.	False Posi.	Posi.	False Posi.	Posi.	False Posi.
Plastridge et al (1935) (2)	(IV) 98.7	13.5								
Fay (1938) (1)	(IV) 96.0	9.0	96.0	41.0	(IV) 98.0	35.0	(II) 37.0	1.0	99.0	60.0
Rowland and Zein-el-Dine (1939) (1)							(I) 61.0	16.0	49.0	34.0
Murphy (1939) (2)					(VI) 96.1	0.4				
Palmer et al (1940) (2)							(II) 40.4	8.1		
Schalm (1948) (2)	(V) 71.0	44.0			(V) 80.0	7.1				

TABLE III (continued)

Authors	Bacterioscopy		Leucocyte Counts 500T or more		Hotis Test		pH Test		Catalase Test	
	Posi.	False Posi.	Posi.	False Posi.	Posi.	False Posi.	Posi.	False Posi.	Posi.	False Posi.
Narayanan and Iya (1954) (2)	(III) 98.9	0.5	85.6	5.2	(IV) 93.4	2.1	(II) 40.8	8.0		
This Paper (1)	(VII) 94.8	42.0	50.9	15.8	(VII) 82.1	35.5	19.6	0.0	54.9	10.6

- (1) --Selective medium
- (2) --Blood-Agar medium
- I --Bromcresol purple
- II --Bromthymol blue
- III --Microscopic examination of Hotis incubated samples
- IV --Streptococcal infection
- V --Micrococcal infection, only
- VI --Strep. agalactiae, only
- VII --Micrococcal and streptococcal infections

considered as positive "an animal in mid lactation if the average count of the last two was over 100,000 per ml."

The level of leucocytes alone cannot be accepted as satisfactory for diagnosis. However, in combination with observation of bacteria in the smears a probable diagnosis may be made. When the samples come from a mid-lactation cow, as in this study, it is safe to consider the finding of 500,000 or more leucocyte per ml. as a good indication of infection.

The results obtained were very low, as compared to the ratios of efficiency generally found in this method. It can be explained due to the following factors: high number of Micrococcus pyogenes isolated because of the use of a selective medium; in sub-clinical and latent infections, the leucocyte increase is not quite as high, and micrococcal mastitis produces a moderately high or not so constant leucocyte increase.

Hydrogen-ion Concentration Test

The pH of the normal milk can vary from 6.4 to 6.8, but is usually 6.5 to 6.6. Physiological variations are found in the colostrum. McIntyre, Parrish, and Fountain (1952) give the average of 6.28 and a range of 6.00 to 6.61. The pH in the milk from cows which are drying off shows a more alkaline reaction.

Mastitic milk generally shows an increase in alkalinity but when a high amount of lactose is metabolized by the



infecting bacteria, the milk may show an acid reaction. The variations of the milk in physico-chemical characteristics are a result of injury and inflammation of the mammary gland, due to the presence of the pathogenic agent.

It seems logical, in this sequence of thinking, that the change toward the alkalinity point can be explained as a result of the abnormal passage of blood elements into the milk. The findings of high cell counts and the presence of abnormal amounts of fibrin could be sufficient basis for this hypothesis. However, Stableforth (1930) studying this possibility, stated: "No evidence has been found to support the view that the abnormal reactions is due to an influx of blood serum; the reaction of the milk shows no constant relation to its antigenic titre in serum-precipitin tests."

Espe and Smith (1952) suggested that the increase in pH values is "probably" due to the presence of carbonates. No evidence was given.

It is known that fore-milk is more alkaline than the subsequent streams. In this study, the fore-milk was used to perform the CMT, thus a low efficiency should be expected.

Modified Whiteside Test

In 1939, Whiteside described a reaction of abnormal milk following the addition of a normal sodium hydroxide solution, as the appearance of a "viscid mass."

Murphy and Hanson (1941) found a close parallelism between the positive Whiteside test and the leucocyte content



of the milk. When samples were scored as 2 +, 100 per cent contained 500,000 or more leucocytes per ml., while samples scored as 1 +, 73.1 per cent had 500,000 or more leucocytes per ml.

Schalm and Gray (1954) applied the test to about 5,000 milk samples and found that 85.14 per cent scored as 1 + with a leucocyte count of over 500,000 per ml. High agreement (99.70 per cent) was found between reactions scored as 2 + and leucocyte counts of over 500,000 per ml.

Dunn, Murphy, and Garrett (1943) concluded that the reaction is mainly dependent on the leucocyte content in the milk, directly or indirectly.

Petersen, Grimmel, and Schipper (1950) postulated that "the reaction is caused by absorption of fibrin on the white cells. . . ." They observed the whiteside phenomenon with charcoal in very fine powder added to blood plasma.

Schalm and Noorlander (1957) analysed the gel clumps seen in the Whiteside reaction and found a fat content of 43 per cent. They were also able to verify with microscopic examination the leucocyte desintegration due to the addition of sodium hydroxide. They concluded that fat has a secondary role in the Whiteside reaction.

Thus, since a high leucocyte count is often regarded as a result of an injury to the epithelium of the mammary gland, the Whiteside test should be an accurate indication of abnormal milk, at least in mid lactation.

The figures found for this test may be considered satisfactory and the considerations made in regards to leucocyte counts, may be applied to this test.

Catalase Test

Koning in 1908, cited by Monlux (1948), was the first to apply the catalase test for the diagnosis of bovine mastitis. The enzyme catalase is found in several kinds of living cells both vegetable and animal. In animal cells, the leucocytes are those in which it is found in larger amounts.

Prouty (1934) found that samples having leucocyte counts of 0 to 100,000; 500,000 to 750,000; 750,000 to 1,000,000 and over 2,000,000 per ml. contained 11.5, 69.0, 92.0, and 96.9 per cent catalase respectively.

Monlux (1948) explained various factors which interfere with bovine mastitis diagnosis using the catalase test.

The catalase test showed an agreement which is about the same as that reported by other workers.

Bacterioscopy

Direct microscopic examination means, of course, the visualization of the milk abnormality in a stained film. This test, which is simple and accurate, shows a general picture of the degree of udder inflammation through the presence of the etiological agent and a significant increase of cells.

Baker and Breed (1920) suggested this procedure for the detection of streptococcal mastitis. Bryan (1935) classified infected milk by the detection of chain of 6 or more cocci, in sample which were enriched by incubation at 37°C for 12-15 hours.

Brown and Bryan (1950) reported on the daily observation for 6 months of one cow infected with Streptococcus agalactiae and another cow with no mastitis, that the test was negative at all times with the non-infected cow and always positive with the infected cow.

The regularity of elimination of Streptococcus agalactiae in the milk gives this test a high score of efficiency.

Bryan and Devereux (1937) using the microscopic examination as a standard, found an agreement of 86.6 per cent with the blood agar plate method.

Fay (1938) reported 96.0 per cent positive results with this test and 9.0 false positives by comparison with bacteriological identification. Using the same type of comparison, Slanetz and Naghski (1939) found 92 per cent and 2.8 per cent positive and false positive results, respectively.

Recently, Narayanan and Iya (1954) also using the same method of evaluation, found a very high figure of efficiency for streptococcal infection as 98 per cent positive and only 0.5 per cent false results.



The bacterioscopic test requires some skill in order to get an accurate interpretation. Some factors which most commonly result in errors are appearance of micrococci exhibiting chain formation; the presence of saprophytic strains often found among the micrococci and less frequent with streptococci; the irregular shedding of the causative agent in the milk and the presence of contaminating bacteria. By combining the observations on bacteria and leucocytes with repeated examination, such factors are controlled.

There are chronic mastitis cases, however, in which infection is so mild that the leucocyte count is within the normal range or, on the other hand, the leucocyte counts found may be due to an expected or physiological increase during early and late lactation. In some instances, the simple presence of bacteria is not enough for an accurate diagnosis and more tests must be carried out to determine the pathogenicity of the strains.

Some morphologic characteristics of the cocci may indicate the presence of saprophytic or pathogenic bacteria but this process is very inefficient. Of course, it is not possible to differentiate coagulase-producing micrococci in stained smears. Since a selective medium for the detection of the coagulase-producing micrococci was employed, it is understandable why poor results were found. If the blood agar plate method was used (hemolysin patterns), the results would be better because 7 coagulase negative micrococci

strains were hemolytic, diminishing in this way the number of false positive scores. If the blood agar plate was the standard method, the per cent agreement would be 76.0.

Also, if bacterial findings were combined with the leucocyte counts, the per cent agreement would be better. But in this study, because the mastitis cases examined were chronic or sub-clinical infections and due in most cases to micrococcal infection, this combination was not to be so conclusive and the agreement of 78.1 per cent confirms this supposition.

Hotis Test

This test was described by Hotis and Miller (1936) for the detection of Streptococcus agalactiae infection. These authors compared the test with blood agar plate isolation and found that of 753 samples tested 95 per cent agreed.

Murphy (1939), also using blood agar plate isolation as a standard, found that 94.86 per cent of the quarter samples contained Streptococcus agalactiae while 53.63, 19.61, and 10.53 per cent contained Streptococcus dysgalactiae, Streptococcus uberis and atypical streptococci infections, respectively. False negative results for the same bacteria were 5.14, 47.37, 80.39, and 89.47 per cent, respectively. Only 0.79 per cent micrococci agreed and 99.24 per cent were false negatives. The false positive reactions for Streptococcus agalactiae were 1.05 per cent with quarter samples.

Miller (1943) using the same method in the comparison of 10,000 to 15,000 milk samples tested at the U. S. D. A. Animal Disease Station, Beltsville, Maryland reported a mean agreement of 85 to 90 per cent positive results. In the same paper reactions due to other pathogenic organisms found parasitizing the cow's udder were described.

By using Edwards' medium for the isolation of streptococci in 4,132 milk samples, Schalm (1944) found the following results: 84.3 per cent positive reactions and 19.3 per cent false positive reactions with respect to Streptococcus agalactiae. In samples yielding Streptococcus dysgalactiae, Streptococcus uberis and atypical streptococci, the Hotis test was positive only 11.6 per cent, suspicious in 46.5 per cent, and negative in 41.8 per cent. With Micrococcus infection, 1.8 per cent were positive, 36.8 per cent were suspicious, and 61.3 per cent were negative.

Later, Schalm (1948) studying the correlation of coagulase positive micrococci and Hotis reactions, found that 80 per cent showed positive reactions and 7.1 per cent showed false positive reactions.

Brown and Bryan (1950) carrying out the modified Hotis test daily for 6 months, on samples from an infected cow having Streptococcus agalactiae and in samples from a non-diseased cow, reported positive results in 100 per cent of the mastitic milk; 15 per cent of false positive reactions were observed in the non-infected animal.

Throop, Swanson and Mundt (1957) demonstrated the efficiency of the Hotis test by comparing it with physical examination of the udder, strip-cup, pH, leucocyte count, microscopic examination and isolation of the etiological agent. The criterion of mastitis diagnosis was shown by direct test combined with 1 or more indirect test. Detection of Streptococcus agalactiae infection by the Hotis test was found in 82 per cent of the samples whereas Streptococcus dysgalactiae and Streptococcus uberis were found only in 50 per cent of the samples. They concluded that the Hotis test is an excellent method for detecting bovine mastitis due to Streptococcus agalactiae but not for Streptococcus dysgalactiae or Streptococcus uberis because of the large number of negative and suspicious results.

The variations of results in scoring Hotis test may be explained by several factors. Thus, the amount of agglutinins present is expected to be important because the formation of flakes or balls on the side or bottom of the tube represent "the alteration of the surface which causes the organisms to clump. . . ." (McCulloch and Fuller--1939). This fact is not only observed with Streptococcus agalactiae but also with Streptococcus uberis and Streptococcus dysgalactiae. McCulloch and Fuller (1939) stated that it is possible to find typical Hotis reactions with any organism which stimulates the production of agglutinins, grows in presence of bromcresol purple, forms clump due to presence of agglutinins and produces acid from lactose.

The number of bacteria present in the sample, the presence of contaminants such as spore-forming bacilli, the existence of more than one pathogen and the ability of the bacteria to ferment lactose are other elements correlated with the readings.

Pounden, et al (1957) stated that "some of the marked variations encountered might well be explained by differences in the suitability of the milk as a medium for the organism. . . ." Some limited evidence for this was presented.

Perhaps these factors make it easier to understand the statement of Plastridge and Hale (1939) when they called the Hotis test "inadequate" for the diagnosis of bovine mastitis due to Streptococcus agalactiae.

The problem of contaminants, which can be diminished by microscopic examination of the milk from the Hotis test tube, is really important. This was found to be true mainly with micrococcal infections because spore-forming bacilli were found 4 times giving a false positive reaction.

The data found in several papers refers mostly to streptococcal infection (Table III). In this work, the Hotis test was used for cocci--both streptococci and micrococci--which resulted in lowered efficacy.

California Mastitis Test (CMT)

The California Mastitis Test is a new bovine mastitis test proposed by Schalm and Noorlander (1957). It is an

indirect test which is based on three known mastitic phenomena: pH modification, Whiteside test and on the abnormal number of leucocytes in mastitic milk.

Schalm and Noorlander (1957) presented data showing a close relationship between CMT score and leucocyte counts of the milk.

The characteristics of these tests were discussed and almost all considerations, advantages and disadvantages, can be applied to this test.

The characteristic readings, however, are principally correlated with the Whiteside phenomenon which is based on the abnormal leucocyte content in the milk which comes from a diseased udder.

Whiteside described this phenomenon as a "viscid mass" and this description agrees with that reported by Puri and Gupta (1955) who stated: "Protein contributes by far more towards the viscosity of milk than any other major constituent of milk." The increase of protein generally occurs in mastitic milk, as well as in early and late lactation.

The surface-active agent used eliminated the secondary role played by the fat content of the milk.

In this work, the CMT was found to be superior to other test except the bacterioscopic examination.

These results were obtained from fore-milk and the characteristics of the material employed eliminated the possibility of large number of false positive results, due

to the presence of physiological leucocyte increase in early and late lactation.

Isolation and Identification

An increase of bovine mastitis due to micrococci has taken place in recent years or, at least, more attention has been paid to it, resulting in more reported cases. Perhaps, because mastitis caused by streptococci is declining as the result of increased use of antibiotic therapy, micrococci infections due to antibiotic resistant strains are often found. This problem has been brought into focus recently. Dolman (1956) commenting on seven decades of research on the micrococci, stated that: "Today, staphylococcal infections appear more prevalent, more virulent, and more unavoidable than they were a quarter century ago, in the pre-penicillin era." This paper refers to human infections but, of course, the same statement can be applied to bovine mastitis.

Schalm and Woods (1953) describing the mastitis complex concluded: "Cocci are the dominant organisms in the bovine udder. Both streptococci and Micrococcus pyogenes are highly invasive for that organ, but with gradual elimination of the former through the use of antibiotics, the latter is assuming even greater significance in the mastitis complex."

These opinions are not generally accepted. Plastridge and Hale (1956) showed that Streptococcus agalactiae

infections have decreased but the micro-organism continues to be considered as the principal agent of mastitis. This evidence is basis on data collected from several years of mastitis research in Connecticut.

These two adverse opinions may be the result of different methods of diagnosis, treatment, and control-programs applied in those areas.

The micrococci. The criterion of judging micrococcal pathogenicity, is based mainly on its ability to secret toxins which have the capacity to lyse the red blood cells of certain animal species and its capacity to coagulate blood plasma of some species. Many papers are concerned with micrococcal hemolysins and so far, four different ones have been accepted.

The presence of hemotoxins elaborated from strains of micrococci, called "Hot-Cold" lysins have been described since 1922 but a differentiation between these two was made by Glenny and Stevens (1935) using specific neutralization. The terms alpha and beta were suggested by them.

The alpha toxin causes total lysis of rabbit and sheep erythrocytes while the beta toxin is a weak lytic agent at 37°C. but becomes rapidly hemolytic at lower temperatures. Hence, this phenomenon is termed "Hot-cold." It may be observed in species in which the red blood cells are not lysed during incubation as occurs with man, rabbit as well as sheep and oxen. Sub-types of alpha and beta toxins have been reported.

The gamma toxin was found by Smith and Price (1938) and can lyse rabbit, sheep, bovine, rat, guinea pig, horse, and human red blood cells.

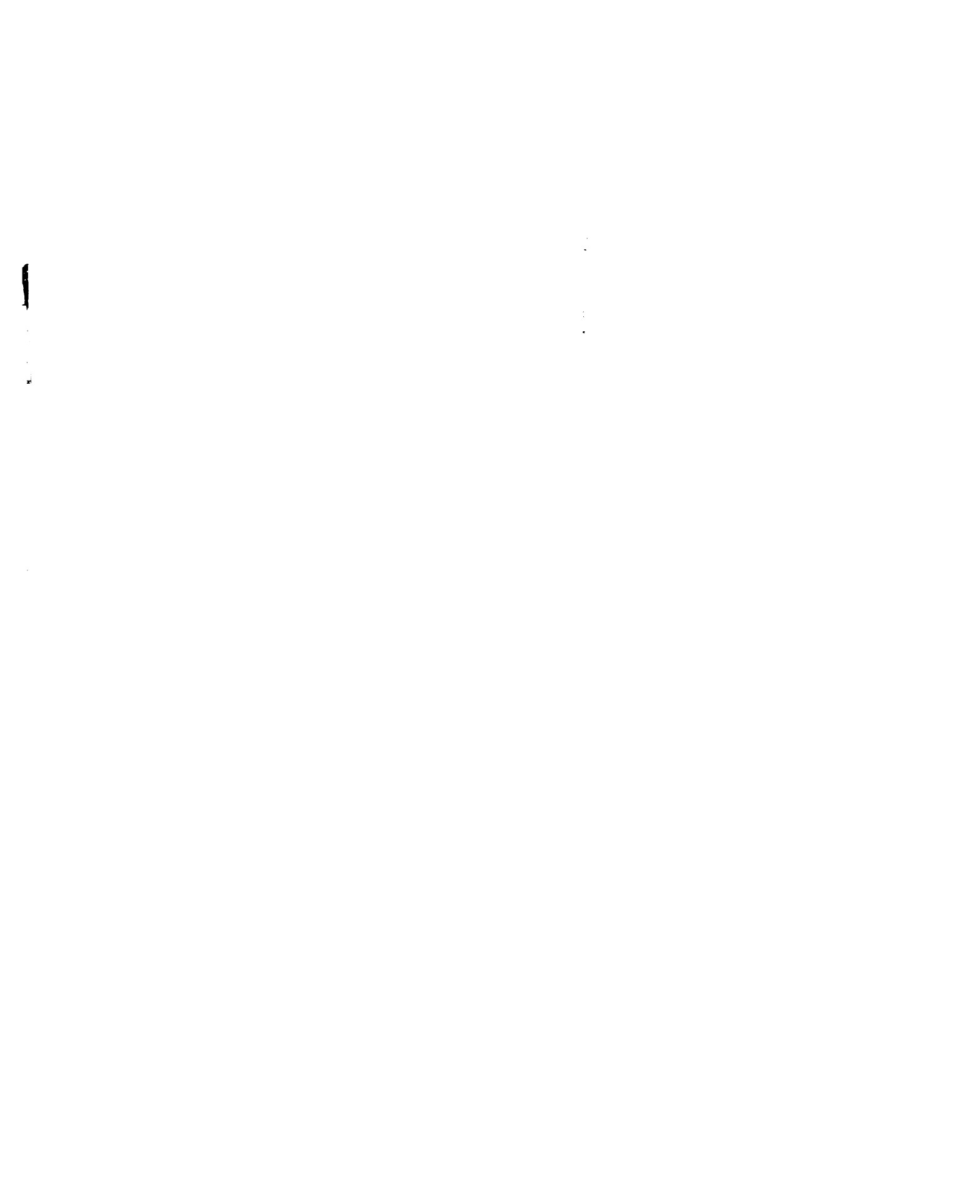
The delta type hemolysins were described by Williams and Harper (1947). These have the capacity to lyse sheep red blood cells protected by alpha, beta or alpha-beta toxins. This hemotoxin is active also against rabbit, human, horse, rat, guinea pig, and mouse erythrocytes.

Elek and Levy (1950) described a new hemotoxin which lyses rabbit and sheep erythrocytes which is called epsilon. It was secreted by a coagulase negative micro-organism. They concluded also that alpha₂, delta and gamma are identical toxins. According to the results and suggestions presented by them, Micrococcus pyogenes produces alpha, beta, delta and epsilon hemotoxins. Only beta hemolysis is incomplete and more than one hemolytic toxins may be secreted by the same strains.

Minett (1936) stated that beta hemotoxin is a characteristic of hemolytic micrococci from animal sources. He later (1937) reported that bovine strains can secrete alpha and beta toxins.

A similar statement was made by Elek and Levy (1950) who also confirmed that beta is often elaborated by animal strains and is not common in micrococci parasitising man.

The ability of some Micrococcus strains to cause the coagulation of blood plasma has been known since 1903 through



original report of Loeb (cited by Smith and Hale--1944). It was only after several years, however, that the presence of this enzyme was proposed as the best single test to judge micrococcal pathogenicity.

Human or rabbit plasma are generally used for this test because of the consistent results obtained. Variations in intensity of results take place with different plasma sources. The explanation for the differences of reactions was given by Smith and Hale (1944) who described micrococcal coagulase as the precursor of a thrombin-like substance formed in the presence of an activator normally found in some plasmas. The failure of plasma to be coagulated is due to the deficiency or absence of this activator. This variation in activator content can be found also in the same species as an individual characteristic.

Smith, Hale and Smith (1947) reported that coagulase is closely related to the pathogenicity of certain strains and described its role in development of infection. It is important in the early stages when the micro-organisms are establishing and starting to reproduce or, in other words, it plays an important role in protecting the micrococci against primary defenses of the host. This is made possible through the fibrin clot formed around the cocci.

The association of hemolysin and coagulase production with pathogenicity has been studied by a large number of workers. Chapman, et al (1934) found that of 690 Micrococcus

pyogenes var. aureus and 1,852 Micrococcus pyogenes var. albus, 88 per cent and 11.9 per cent were coagulase positive, respectively. With Micrococcus pyogenes var. aureus, they reported: 78 per cent of non-hemolytic and coagulase negative strains did not kill rabbits in 10 days; 100 per cent of coagulase and hemolysin positive strains killed rabbits in less than 10 days. With Micrococcus pyogenes var. albus, they found: 77 per cent of 17 hemolytic strains did not kill rabbits in 10 days and 88 per cent of 16 coagulating strains killed in less than 10 days.

Williams and Harper (1946) tested 100 coagulase positive strains and reported that 93 were alpha hemolytic and 2 were beta hemolytic. The strains were, presumably, from human sources.

In comparing laboratory methods accepted as evidence of micrococcal mastitis, Plastringe, Weirether and Williams (1938) found that the coagulase test was more nearly correlated with the micrococcal pathogenicity than the other tests used. They reported hemolytic properties in 87 per cent of the coagulase positive strains and 5 per cent from the coagulase negative bacteria.

A high frequency of alpha-beta and beta hemotoxins to bovine red blood cells was found by Schalm and Wood (1953a) who tested 2,084 hemolytic strains, almost all from bovine mastitis, with a coagulase positive score of over 99 per cent. Out of 209 micrococci non-hemolytic or weakly hemolytic

but showing no beta toxins, 17 were coagulase positive. They stated that "for practical applications, manifestation of beta toxins should be sufficient evidence that the Micrococcus is Micrococcus pyogenes." Coagulase tests should be used as a decisive test in questionable cases. This can be accepted because coagulase production is a special property of Micrococcus pyogenes.

Slanetz and Bartley (1953) studied about 9,000 strains of micrococci from mastitic milk and concluded that: (1) They may be alpha-beta, beta or delta hemolytic to sheep, cow, and rabbit red blood cells. In most cases they are the alpha-beta type. (2) With the exception of weakly toxigenic strains which are coagulase negative, these micrococci are usually coagulase positive. (3) Leucocyte counts are not significant for the diagnosis of chronic mastitis infections due to micrococci.

Boake (1956) presented conclusive evidence on the real importance of coagulase in micrococcal pathogenicity. He found that rabbits actively immunized with coagulase were more resistant to challenge with coagulase positive and alpha hemolytic strains (mean survival time of 9.3 days) than those immunized with alpha toxoid (mean survival time of 1.3 days). Unprotected individuals had a mean survival time of 1.5 days. On the other hand, rabbits immunized with coagulase and later challenged with a coagulase negative strain showed no resistance and died 2.5 days after

inoculation. The same survival time was found in the control group. Based on mice experiments, Boake (1956) found that "in vitro" inhibition of coagulase activity appeared to diminish the pathogenicity of micrococci.

Rammelkamp, Jr., et al (1950) were able to demonstrate anticoagulase--an antibody specific for coagulase--in 11 of 17 monkeys injected with cell-free coagulase.

Although coagulase is an important factor in pathogenicity, it does not appear to be the only mechanism for this condition. There are authors like Deubler and Cole (1956) who believe that coagulase and hemolysin are not indicative of micrococcal pathogenicity. From 12 cases of acute micrococcal mastitis they isolated 8 strains which were both coagulase and hemolysin negative.

From a comprehensive review of the literature on this subject, one can see that a definite method for evaluating micrococcal pathogenicity has not yet been found. Coagulase production, however, appears effective in this respect than hemolysin activity. Analysis of studies in this subject reveal that many controversies are brought about because of the differences in the blood employed, both their sources and concentration; the sources and dilution of blood plasma and finally the different technique used.

Due to these difficulties other methods have been suggested for indicating pathogenicity such as the number of antigen-antibody flocculation lines formed on a plate

(Howard--1954) and phage pattern (Fisk--1942; Howard--1954, and Edwards and Rippon--1957). In this study, coagulase production was used as an indicative test of the micrococcal pathogenicity.

Zebovitz, Evans and Niven (1955) employed a Tellurite-Glycine Agar medium to isolate coagulase producers micrococci. They compare the new medium with Chapman-Stone medium and Phenol Red Mannitol Salt Agar and reported significantly superior results with the improved medium.

Innes (1953), using Ludlam's medium for plating milk samples, reported the isolation of 5 coagulase positive micrococci from 24 milk samples cultured. The same samples plated on Blood-Agar yielded no coagulase-producers due to the high interference of contaminating bacteria. Samples were enriched in special selective broth before incubation.

Velilla, Faber and Pelczar (1947) using Phenol Red Mannitol Salt Agar medium (PRMS) and Staphylococcus medium 110, were able to isolate 90 strains of micrococci on the first medium and 137 Micrococcus strains on the latter medium, from a total of 270 quarter samples. From 90 of those strains isolated using PRMS, only 18 were coagulase-producing micrococci while from the 137 isolated on the medium 110, 31 were coagulase positive.

The results found by using Tellurite-Glycine Agar medium (83 per cent agreement) were superior to those obtained by Innes (1953) on Ludlam's medium and by Velilla,

Faber and Pelczar (1947) on PRMS agar or Staphylococcus medium 110.

The criterion used to identify pathogenic Micrococcus taking its ability for coagulating blood plasma as a simple and single test, is actually superior to the production of hemolysins. Coagulase is found in almost all pathogenic strains of Micrococcus pyogenes and it is believed to be a specific characteristic. On the other hand, hemotoxin production is a less constant occurrence in pathogenic micrococci and it has been found to be elaborate by coagulase negative strains. However, in this study, a total agreement was found between alpha-beta hemolytic and coagulase-producing micrococci.

By using the Tellurite-Glycine Agar medium, it is possible to isolate a coagulase producers micrococci strain in 24 hours.

Catalase production by cocci. By testing for catalase in cultures of cocci, one has an easy way of screening out certain streptococci (short chain formation). It is generally agreed that catalase production is very constant in Micrococcus, although Lucas and Seeley (1955) reported the finding of a negative catalase strain which was strongly coagulase positive.

The data obtained in this work confirms earlier observations on the catalase test.

The streptococci. The selective medium of Edwards was used to culture streptococci. From the color and size of colonies, streptococci, Pseudomonas and coliform organisms can be recognized but the micrococci were inhibited. In this medium the selective agents are crystal violet and esculin. Identification was made on the basis of the following tests: CAMP test described by Murphy, Stuart and Reed (1952), catalase production, esculin, mannitol and inulin breakdown, hydrolysis of sodium hippurate and hemolysin production.

The CAMP test is an application of the lytic phenomenon found by Christie, Atkins and Munch-Pettersen (1944) in which Streptococcus agalactiae, hemolytic or non-hemolytic, secretes a lytic product that can totally lyse sheep or oxen erythrocytes previously altered by a micrococcal beta toxin.

Wilson and Salvin (1950) included some micrococci as CAMP positive but confirmed the fact that all the Streptococcus agalactiae tested were positive.

However, Murphy, Stuart and Reed (1952) reported that 96.6 per cent of 323 strains of Streptococcus agalactiae produced CAMP reactions, whereas 85 per cent of 222 strains of Streptococcus uberis and 100 per cent of 49 Streptococcus dysgalactiae gave negative results.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The California Mastitis Text (CMT) was applied to 70 milk samples and its value as a new method for the detection of bovine mastitis was compared with 7 well known tests.

The CMT, which is an indirect test, compared favorably to leucocyte counts, pH, Whiteside test, catalase test, and Hotis test but not to the bacterioscopic method.

The bacteriological finding, which was used as the standard test, was made on Edwards' medium and on the new Tellurite-Glycine Agar medium. This medium, which is a modification of Ludlam's medium, proved to be very efficient for isolating coagulase producing strains of micrococci.

Coagulase and hemolysin were studied and their value as indicators of micrococcal pathogenicity was discussed.

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