Mastitis—Laboratory Tests and Their Interpretation*

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The American consuming public is losing huge amounts of milk and the American dairy farmer is losing an astronomical number of dollars every year through the inroads of mastitis. Today, when it is so vitally important to keep every milk-producing cow in good health so that she can make the maximum amount of milk on the allotted hard-to-get feed, the subject of its control becomes urgently important.

Recognizing the timeliness of this problem, the Extension Department of the College of Agriculture here at the University of Vermont has recently inaugurated a program. Under it, sound preventive herd management practices, as well as the economic importance of mastitis to the herd owner himself, will be emphasized. Any success that may be had will make the job of everyone interested in milk quality control that much easier.

Before such a program can get very far, sound laboratory methods will have to be used to locate for sure the offenders, and determine the status of infection and probably the type of organism causing the trouble, in order to intelligently segregate, treat, or dispose of such animals.

Selection of diagnostic methods is extremely important. They must be adequate, relatively simple, and capable of as widespread application as facilities and personnel will permit.

No known single test can do this job.

The attempted determination of the comparative accuracy of various tests commonly used to aid the diagnosis of mastitis has been a popular pastime for a number of years. The results in some cases have been rather disappointing, largely because different yardsticks were used, and different skills, in both manipulation and interpretation, were brought into play.

Although we are probably primarily interested in *Streptococcus agalactiae* as the predominant cause of infectious chronic mastitis, we cannot ignore the possibility of the organism which we find being any one of several others.

Mastitis is not a "one bug" disease like typhoid, diphtheria, etc. Organisms, other than *Streptococcus agalactiae*, which are not at all uncommon are: *S. dysgalactiae*, *S. uberis*, *S. faecalis*, certain staphylococci and members of the coliform group. There are others which have been mentioned in the literature from time to time, but these are probably the most important.

It is quite generally agreed that early diagnosis increases the efficiency of herd disease control. (Segregation, disposal or treatment.) This depends to some extent upon the type of organism causing the trouble as well as the degree of infection.

Before some of the more common and most useful tests are described and discussed, a word should be said about sampling as it is vital to the success of any laboratory test.

1. Samples should preferably be taken just before milking but never within at least two hours after milking.
2. The sample taker should wash and disinfect his hands between cows.
3. The udder and teats should be washed...
with a chlorine solution of about 200-400 p.p.m.—or other acceptable germicide—immediately prior to drawing the sample, giving particular attention to the end of the teat.

4. The first two streams from each quarter should be milked into a strip cup—this helps to avoid contaminating bacteria which may have gained entrance into the teat in the interval since last milked. At this time it can also be observed whether the milk from any quarter is flaky, stringy, or otherwise abnormal.

5. Each sample bottle or container should be carefully identified. Carelessness about this detail has been known to cause confusion and embarrassment.

**Physical Examination**

The physical examination of the udder is not, of course, a laboratory method and therefore does not properly belong to the topic assigned to me for discussion. However, it cannot be passed over without mention. It is a diagnostic aid of potentially great importance whose value varies in direct proportion to the skill and judgment of its user.

After enumerating eight important steps in the proper observation and palpation of the udder, Dr. Udall (14) in his book, "The Practice of Veterinary Medicine," says, "While the technic may appear to be simple, skill in the classification of udders is acquired only after long practice. It includes not merely the art of detecting indurations, but experience and judgment in reaching a decision upon all the evidence available." He further says, "Conclusions based on a milker's statement that an udder is normal, or on a superficial examination of the udder or on an examination by one who lacks training in palpation of the udder is unreliable."

Many people believe that udder palpation is one of the most reliable methods for determining whether a cow actually has mastitis or not. They hold that the seat of this disease is in the udder and not in the milk; that significant alterations in the udder tissue can be disclosed by competent manual examination and therefore that the mere finding of unidentified streptococci in the milk unaccompanied by evidence of tissue involvement does not constitute positive diagnosis of disease. It is difficult to quarrel with this view. On the other hand, affected cows do sometimes recover, either through treatment or even automatically. Under these circumstances there will be a varying amount of scarred tissue, etc., of a permanent nature. How then can a cow that has mastitis be differentiated from a cow that has had mastitis? This is mentioned only to emphasize the point that physical examination alone, even though expertly done, may not be enough to establish beyond doubt, an infected udder in the absence of adequate confirmatory laboratory tests.

It should be said that the thorough veterinarian often uses the strip cup, brom thymol blue test, and laboratory examination of the milk to increase the efficiency of his diagnosis.

**Strip Cup Test**

The strip cup test is designed to detect abnormal milk in the barn during milking rather than after the milk has been taken to the laboratory.

There are several types of strip cups that may be used. One of the most popular consists of a heavy tin cup holding a pint or more which is fitted with a removable top about 1½ inches deep. One-half the bottom of this section is made of fine wire mesh (100-120 squares to the inch), the other half made of tin. A piece of fine black cloth stretched over the top of a tin can may be used for the same purpose. This test is best made when the udder is filled with milk just before regular milking time. The procedure is to draw the first two or three streams from each teat directly on the sieve or black cloth and carefully examine for flakes or clots. The top
should be removed and rinsed in water after each test. Many believe that the routine use of the strip cup can be of great value in revealing early an acute inflammation or a flare-up of a chronic case. Obviously a single survey of a herd is of little value as many chronically infected cows do not at all times give the type of milk detected by this method. However, it should be noted that flakes may be present in milk of abnormal color as a transient condition after injury. Thin, watery milk of abnormal color (brownish, etc.) may be given in which no flakes can be found. This type of milk can be easily spotted if set up in test tubes and compared with normal milk.

Use of the strip cup should be made a part of every milking routine, especially if a milking machine is used, without after stripping. This affords the dairyman daily contact with the udder and makes it possible for him to pick up many incipient cases which might otherwise be passed over for a long time. However, hurriedly milking into the strip cup without careful examination of results is a waste of time and energy.

Care should be taken in the disposal of the contents of the strip cup—never pour it into the gutter.

Finding of clots or flakes are usually confirmed as indicating mastitis but their absence does not necessarily indicate freedom from trouble.

**Brom-thymol-blue Test**

The brom-thymol-blue test is a semilaboratory test which can be and often is performed in the stable, either by a veterinarian as a confirmatory test in connection with physical examination of the udder or by the dairyman himself.

When it was first introduced it was widely heralded as the answer to the prayer for a simple, accurate test whose results were nearly infallible. It received world wide study and was found to have certain limitations and pitfalls. There is supporting evidence for the theory that active infection induces an infiltration of the blood serum (and perhaps other fluids) into the udder which carry leucocytes whose function, as scavengers of the blood stream, is to halt or control further invasion of the infecting organism and to aid in the repair of damaged tissue. At any rate, blood serum is known to be on the alkaline side while normal milk is slightly acid. This test is a measure of acidity or alkalinity (hydrogen ion concentration or pH as it is commonly spoken of).

One way of performing the test is draw 5 ml. of the milk from four separate quarters into four separate tubes. This test is useless when applied to pooled milk samples. Next add 1 ml. of the brom-thymol-blue solution of the proper strength and which has been adjusted to near neutrality. (Made by dissolving 1 gram of the powder in 125 ml. of alcohol—either methyl or ethyl but not denatured—this is then made up to 500 ml. with distilled water. It is then adjusted to near neutrality by the addition of small amounts of normal sodium hydroxide.) Each tube is carefully inverted and examined for color changes.

1. A greenish-yellow denotes a milk more acid than normal.
2. Yellowish-green usually denotes a normal milk.
3. Light green denotes a slightly more alkaline than normal milk and therefore suspicious.
4. Dark green to greenish blue denotes a distinctly alkaline milk which is usually indicative of mastitis.

Occasionally a bright yellow or orange reaction will be encountered which usually is found to come from a cow suffering from an acute case of mastitis.

I have found it desirable to examine and compare samples from the four quarters at the same time—differences are more easily noted. Rarely are all
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four samples of exactly the same shade due to varying amounts of pigment carrying fat. I have also found color standards to be of but little value except to give a rough idea of what to look for.

In the course of testing several thousand samples of milk it has been noted that when a suspicious reaction is found repeatedly in the same quarter or quarters that later if that cow definitely develops mastitis it will almost invariably be in those quarters.

Care should be taken when using a single set of four tubes for herd testing to avoid using tap or well water to rinse them between tests. This type of water is almost always distinctly alkaline and might lead to erroneous conclusions in borderline cases. Either use distilled water, rain water, or normal milk for this purpose.

Milk from cows early or late in lactation may give misleading results and cannot be depended upon.

Positive results with this test are said to be indicative of mastitis in as many as 90 percent of cases while negative results do not necessarily mean that the cow is mastitis-free.

This test is much more valuable as a routine test, frequently performed, than as a survey test.

Other dyes which are sensitive to reaction changes are sometimes used for this test such as brom-cresol purple. However, the dye of choice now seems to be brom-thymol-blue, sometimes called thybromol.

Modified Whiteside Test

The modified Whiteside test is one of the newer tests. A modification proposed by Murphy and Hanson (9) is very practical and can be run in the stable with a minimum of equipment. All that is needed is a glass plate or plates ruled into two-inch squares; a dark background; a glass stirring rod; two droppers and some normal sodium hydroxide solution. To perform the test one drop of the normal NaOH is added to 5 drops of the fore milk of each quarter and stirred for 20 seconds. Normal milk remains unaffected. Mastitic milk becomes coagulated in rough proportion to the degree of infection. A viscid mass is quickly formed which, except in the worst cases, quickly breaks up into a slightly opaque fluid and a precipitate composed of many rather large particles.

It has been reported that increased accuracy can be obtained by refrigerating samples over night, also that where fresh samples are used increasing the NaOH used to two drops increases the accuracy of this version.

In explanation of what happens in this test, Dunn, Murphy, and Garrett (3) have recently postulated the theory that the protein material of leucocytes in mastitic milk reacts with the sodium hydroxide to form a gelatinous mass similar to that which is formed by the action of sodium hydroxide on the nucleic acid obtained from animal cells.

We have used this test on several hundred samples and have found that the results roughly reflect the leucocyte count.

Catalase Test

The catalase test is also based on the presence of leucocytes which secrete varying quantities of the enzyme catalase which in turn is able to release hydrogen gas from hydrogen peroxide when added to the sample being studied. This becomes immediately evident by the evolution of minute gas bubbles. There are many ways of performing this test all the way down from a more or less exact measurement of the amount of gas to rougher qualitative determinations. We have found the simplest method is to place a drop or two of the quarter sample on a glass plate with a dark background and mix with a drop of 6–9 percent hydrogen peroxide. Mastitic
milk supposedly produces bubbles which are not evident in normal milk. A small hand lens greatly aids in this examination.

Rosell (11) suggested adding 1 ml. of 6 percent peroxide of hydrogen to the brom-thymol-blue test after color comparisons have been made. The tube is then mixed by careful inversion and closely observed for the presence of the gas bubbles which quickly form in the presence of leucocytes. He says that the results closely conform to the brom-thymol-blue color changes. This would appear to be a laboratory procedure.

**Chlorine Test**

The chlorine test is regarded by many workers as a very delicate and valuable test and an accurate indicator of the presence of fibrosis in the udder. It has been found that although the chloride content of normal milk is somewhat variable, it usually runs between 0.09 and 0.14 percent chlorine. Mastitic milk usually runs significantly over the top figure. There are many ways of obtaining this information running all the way from methods which give the exact percentage of chloride down to the so-called field tests which merely determine whether there is more than the normal amount present. We have used Hayden's field test with success. The reagents consist of an accurately made silver nitrate solution (1.3415 grams of the chemically pure product in 1,000 ml. of distilled water) and a 10 percent solution of potassium chromate. The test is performed by measuring an accurate 5 ml. of the silver nitrate solution into a small test tube. Add 2 drops of the chromate solution. A red color develops at once. Then add an accurate 1 ml. of the quarter sample of milk. Carefully invert to mix. A yellow color develops in one minute or less if the chlorine is 0.14 percent or over. Normal milk remains red. Even this field test has been found to be a laboratory procedure and is not recommended for rapid field work. Much depends on the care with which the silver nitrate solution is made up.

Both the catalase and the chloride tests are open to the criticism of being over sensitive. That is, they produce too many false positives.

**Microscopic Examination**

It is hardly necessary to describe the details of this method for it has been an important and serviceable tool of the dairy industry for more than 30 years. It early became an important aid in mastitis diagnosis. I used it for that purpose twenty-five years ago. However, there are a few things about its application that will be briefly discussed.

The source of any given sample determines, to an important degree, the dependability of ensuing results.

The important point is, however, to obtain a sample and so handle it that the maximum of desirable information may be had from it. In the search for mastitis, samples are often taken from the weigh can, individual cans, pooled quarter samples and from the individual quarter. They are examined before and after incubation. Microscopic examination of unincubated samples from any source are almost a waste of time, except where cell counts alone are desired. Too many significant cases will be overlooked.

It is realized that weigh can samples are widely used for rapid survey purposes—largely as a matter of expediency and not because the user actually believes that the results are too revealing. The presence of long chain streptococci with or without many leucocytes is commonly accepted as proof of mastitis in the herd from which the sample comes. It should be remembered that such samples are nearly always contaminated, that con-
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Taminants are the first to multiply under incubation, and that they often appear as long chain streptococci indistinguishable in smears from mastitis streptococci. It is probably true that from grossly diseased herds the milk may be sufficiently infected to show mastitis streptococci in the smear from weigh can samples. However, the fact that as a result of such an examination, infected cows are later found in the indicated herds proves nothing because it would be difficult indeed to find a herd in this State or any other state in which one or more mastitic cows could not be located.

Examination of any of these samples is made in the search for two things:

1. Long chain streptococci or possibly staphylococci or the coliform types.
2. Leucocytes.

Assuming that samples of individual quarters have been aseptically drawn into sterile bottles and incubated for 12 to 16 hours at about 37°C and that the smears have been properly prepared—then what?

The first problem that arises is—what are long chain streptococci? In other words, when do short chain streps become long chain streps? Investigators do not all agree on this. Figures from 4 to 10 units per chain are found in the literature as the dividing line. I am not prepared to say what the correct figure should be nor how important it is. However, I do feel that where there is any question there should be verification.

There may be and usually are other cells of less significance than leucocytes found in milk. I am inclined to think that cell counts are more often made than leucocyte counts.

In speaking of cells, those originating in body fluids and tissue are meant and not the invading bacterial cells.

In regard to leucocyte numbers—what is normal? Figures are found all the way from 50,000 to 1,000,000 and over. According to Seelman (13) the normal fluctuation in numbers of leucocytes are so great and the sensitivity of the mammary glands so variable that the establishment of a fixed normal would only lead to error. Nevertheless, informed opinion in this country, resulting from the study of thousands of samples, most often places the figure at 500,000 per ml. as indicating an udder abnormality and possibly mastitis—certainly suspicious as possibly indicating incipient infection.

Transient cell counts of several millions have been observed with no other evidence to be found. The cell count is normally high in late lactation. Occasionally it may be high as a result of disease other than mastitis—such as winter dysentery.

There is little doubt about a microscopic picture of millions of leucocytes per ml. and extremely long chains. It is the borderline cases that are the headache.

If the herd owner is to be helped in his effort to control this disease, correct interpretation of these borderline cases is of importance. They may be the ones which can be greatly helped by treatment if discovered early. If they persist with or without treatment it may be that they are carriers and thus a menace to their mates—even though they themselves are able to hold the disease in check.

Carefully taken and incubated quarter samples are to be preferred for microscopic examination. Some laboratories that are doing a large volume of routine work have been forced to use incubated pooled udder samples. Undoubtedly an occasional lightly infected cow will be passed over if this type of sample is resorted to.

Hotis Test

The Hotis test is partly chemical and partly bacteriological in its disclosures. This test was first introduced in 1936 by Hotis and Miller (5) of the U.S.D.A. It involves the addition of 0.5 ml. of a sterile 0.5 percent aqueous solution of brom-cresol-purple to 9.5
ml. of an aseptically drawn quarter sample of milk. It is sometimes used on a composite udder sample; under these circumstances it is customary to double the quantities of both dye and milk sample. It cannot be successfully used on weigh can samples due to the inevitable contamination by organisms which obscure the results obtained. According to Miller (7), when the dye and milk are first mixed, a purple color results. The reason for this color is that normal milk is very slightly acid and this pH is in the upper range of the dye which runs from pH 5.2 which is yellow to pH 6.8 which is distinctly purple. Since the pH of normal milk is slightly acid such samples will be a sort of a pale grayish-purple or dove color. Samples from an infected quarter are more alkaline and have a distinct to deep purple shade. By noting the color at this time, as suggested by Cone and Grant (2), considerable information can be obtained about the physical condition of the quarter or udder. The samples are then incubated at 37° C. for 20-24 hours. At this time the color of the column of milk varies from dove gray and olive drab to canary yellow. In general, samples containing Streptococcus agalactiae, flakes, or clumps of a whitish material will be noted clinging rather tenaciously to the sides of the tube as well as a coarse flocculent sediment in the bottom of the tube. The color of the flakes varies somewhat according to the type of streptococci. Dr. Little (6) has noted that in mild infection the yellow deposits may appear only in the cream line or at the bottom of the tube. In addition, it has been found that some, though not all, types of staphylococci form rust colored colonies on the sides of the tubes. Because these usually are slow acid formers there is less change in the color of the milk.

To counteract the unfavorable action of contaminating bacteria in obscuring positive changes in the test, certain agents have been added whose function it is to inhibit the growth of these contaminants without interfering with the growth of the streptococci. One such agent that has been suggested for this purpose is sodium azide. Several workers have found it very useful for this purpose. However, Miller (7) reports that the changes are not entirely typical in all cases and that 48 hours may be required for positive results to occur. Also that staphylococci do not grow well in the presence of sodium azide. Consequently some information that might be obtained from the test are thereby lost. It might be added at this point that some workers now feel that staphylococcal mastitis is somewhat less than uncommon and must be reckoned with more in the future. According to Schalm (12), "The staphylococci are usually involved in severe forms of acute mastitis and gangrene of the udder, but may also be the cause of a mild chronic mastitis. Knowledge is limited concerning the origin, mode of spread, and methods of control of this type."

Miller (7) also reports that since the test has been in use at the U.S.D.A. Animal Disease Laboratory, between 10,000 and 15,000 samples have been examined in comparison with blood agar plate cultures, and has maintained an average agreement of from 80-90 percent.

Murphy (8) has reported that, "application of the test to 753 samples of milk in conjunction with cultural examination in blood agar showed them to be in perfect agreement for 95 percent of the samples." Others have reported a much lower correlation—sometimes as low as 30 to 50 percent. However, it seems to be highly regarded in some quarters—especially by those who have used it most and understand it best. It is used in some places as a screen test from which samples from suspicious or positive tubes can be smeared and examined microscopically.

If more information is needed as to
whether or not the causative organism is *Streptococcus agalactiae* it can be plated in plain blood agar or streaked on the aesculin-gentian-violet blood agar described by Edwards. The system suggested by Plastridge for more definite identification uses loop transfers from samples showing either streptococci or more than 500,000 leucocytes per ml. to ox blood agar or Edwards medium which he considers adequate for the detection of *Str. agalactiae*. He adds that in ordinary routine work growth observed on Edwards medium alone is considered adequate except under special conditions. Many workers believe that Edwards solid medium is more useful than the plain blood agar because, in addition to indicating whether or not the organisms are hemolytic it yields other desirable information.

Dr. Little has reported that where it is advisable to use more critical tests, Edwards liquid medium has certain advantages over plating in blood agar. It is simple to prepare—containing meat extract, glucose, crystal violet and sodium azide. The function of the crystal violet is to inhibit the growth of the staphylococci while sodium azide prevents the development of coliform organisms. He suggests using it in conjunction with the Hotis test, modified by the addition of sodium azide. After incubation for 16 to 18 hours, a loopful (0.01 ml.) of gravity cream is transferred to the selective medium and the samples incubated for the completion of the Hotis reaction. The development of *Str. agalactiae* in the liquid medium as a flocculent growth with the clearing of the supernatant fluid is of diagnostic significance, for usually other types of streptococci cause turbidity of the broth.

Dr. Little also reports that extracts from bovine streptococci can be prepared from the sediment of Edwards' liquid medium for serological identification.

For exact typing it is necessary to either use a serological classification or an even more complicated and time consuming biochemical study either one of which is out of the question for large scale work.

Up until recently, wherever efforts have been made to control mastitis, work has been concentrated on the latent (or sub-acute or chronic) type caused primarily by *Str. agalactiae*—this for two reasons:

1. It is by far the most prevalent.
2. It is probably the most infectious.

It has been reported that in herds where they have had considerable success in eliminating *Str. agalactiae*, other types such as staphylococcic and coliform mastitis have made their appearance. Provision for their recognition should be considered. With this in mind the desirability of including growth inhibitants in some of the suggested media and tests should be reviewed.

**Vermont Program**

Now as to the Vermont program. Obviously unrestricted laboratory service is out of the question. However, it is hoped to eventually set up several demonstration herds. The idea is to keep the number down to a point where adequate laboratory supervision can be maintained. It is hoped thus to be able to indicate by precept what can be done with sound herd management plus a certain amount of laboratory assistance, materially to lower the incidence of mastitis in Vermont dairy herds.

The tentative diagnostic procedure will be as follows: In addition to the routine use of the strip cup, physical examination of the udder and the brom-thymol-blue test, all of which will be performed in the stable, aseptically drawn quarter samples (or perhaps pooled udder samples) will be taken for the laboratory. In cases where they cannot be promptly transported, Bryan's suggestion as to the use of inhibitory agents, such as brilliant green, and sodium azide, will be tried.
The Hotis test will be run on all samples. Those which are either positive or suspicious will be examined microscopically and transferred to either Edwards’ blood agar medium or the plain blood agar, as an additional check.

On the basis of this information it is hoped to recognize incipient mastitis early and thus facilitate more intelligent and fruitful segregation, treatment or disposal.

It should be remembered that men of wide experience in this field do not agree on the details of proper procedure. Therefore, about all that can be done is to adopt a routine with which, it is believed, dependable results can be obtained. Experience here and elsewhere will inevitably lead to changes. An effort will be made to keep an open mind and adopt only those methods which, in our opinion, are most applicable to this particular problem and are best substantiated by sound research.

LITERATURE CITED