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Adaptations and Limitations of Ring Test for Bovine Brucellosis

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K. L. TALLMAN and H. A. HERMAN

INTRODUCTION

Monetary loss from Brucellosis is approximately $90,000,000 annually in lowered milk production and calf losses, according to U. S. Livestock Sanitary Association estimates, in spite of advancement in methods for its detection. By virtue of its simplicity, low cost of application, and speed, the ring test is meeting with increasing favor as a brucellosis detector.

The ring test is conducted by adding two drops of a stained antigen to 2 ml. of milk in a small test tube. The sample is mixed and incubated at 37°C for 45 minutes to one hour, or left at room temperature for one and one-half hours.

Although extensive studies have been made on the ring test, need for further research is indicated by limitations which still exist. Limitations include: (a) False positive reactions found on pooled milk: (b) unreliability on individual cow samples; (c) inability to test non-lactating animals; and (d) inaccuracies caused by abnormal milk. This study has been undertaken in an attempt to determine causes of these limitations and to gain a greater insight into potentialities of the ring test.

PREVIOUS WORK

Evolution of the Ring Test

The whole milk ring test for brucellosis detection in cattle was first described by Fleischhauer (1937) and Fleischhauer and Hermann (1938). A series of papers were subsequently published during the next two years by German workers on the subject, including articles by Smitmanns (1938), Hermann (1939) and Seelemann and Mantovani (1940). In each case, comparisons were made of the ring test and the slow whey agglutination test. All reported close agreement between the two tests with the ring test giving more positive reactions upon herd milk and about equal to the whey agglutination test upon individual cow samples.

Early Developments

Fleischhauer (1937) concluded from his results in testing herd milk that only those reactions which occurred within 20 minutes could

be considered positive as the majority of all herd samples would show ring reactions after 40 minutes of incubation. One year later, Smitmanns (1938) stated that reactions on herd milk should be considered positive if the reaction started within 25 minutes after the beginning of incubation, and if there was a 2 to 4 mm. wide blue-violet ring clearly separating the underlying white milk at the end of 2 hours.

Fleischhauer and Hermann (1938) concluded from their experiments on composite samples that the beginning time of the ring test reaction was more important than the end. Seelemann and Mantovani (1940) reported that even definitely negative samples had a narrow, bluish violet ring at the end of incubation for 1 hour, although the milk column showed no loss of color.

Bruhn (1948), in an extensive review, pointed out that results of these earlier workers showed that the antigen used was relatively non-specific, giving rise to numerous false positive reactions, particularly with incubation periods which exceeded 30 minutes. These unreliable results were attributed to a Schern-Gorlisch type reaction.

Schern-Gorlisch Reaction

The Schern-Gorlisch reaction, as described by Schern (1936), is a test designed to differentiate between heated and non-heated milk. The test is carried out by adding one drop of a 2 percent suspension of red blood cells or one drop of a 1 percent solution of bone charcoal to 1 ml. of milk. With raw milk, the red blood cells or bone black, adhere to fat globules and are carried to the surface, forming a correspondingly colored ring. Milk which has been heated to 64° to 65° C. for 30 minutes loses the ability to carry the indicator to the surface, which is thought to result from denaturation of the protein layer surrounding the fat globule. Variations in fat content, size of fat globules, and the nature of the protein layer surrounding fat globules have an effect upon accuracy of the Schern-Gorlisch reaction. Therefore, the test is most reliable when performed upon pooled milk. As suggested by Bruhn (1948), many of these same principles apply to the ring test — particularly the effect of heating.

Development of Specific Antigen

Use of reliable antigen was apparently not employed until 1943 when Norell and Olson (1943) published results indicating the antigen they used was the most specific of any produced up to that time. They were able to incubate samples for a period of 50 minutes without obtaining a large number of non-specific reactions, thus eliminating to a great extent the misleading Schern-Gorlisch reaction. They found the ring test to be much more specific on herd samples than the slow whey agglutination test. In a later study, however, Norell and Olsen

(1943) reported a larger number of doubtful ring test reactions indicating the use of less specific antigen than they previously had employed.

Winther and Hansen (1943) experienced difficulty with non-specific reactions. They reported that incubation of ring tests for more than 30 minutes gave rise to false positive reactions. They found the ring test gave 6.4 percent more positive reactions on herd milk than the slow whey agglutination test and about double that number on individual cow samples. They concluded that non-specific reactions occur only among weakly positive and doubtful tests, whereas strongly positive and clearly negative reactions must be considered safe and specific.

It was not until 1944 that proof of the specificity of the ring test was definitely established. Using antigen prepared and tested by Bruhn (1944), Seit and Leth Jorgensen (1944), working on behalf of the Danish State Veterinary Directorate, proceeded with ring test examinations at Bornholm. In a comparison of ring test reactions on 3,362 herds with individual cow blood test reactions, they reported 96.2 percent overall agreement between the two tests. Their results showed the ring test 86.6 percent efficient in locating infected herds. An incubation time of 40 minutes was employed for ring testing. When their results are reviewed, this indicates the use of specific antigen.

Bruhn, whose results were published at the same time as those of Seit and Leth Jorgensen, reported on the relative specificity of ring tests when comparative incubation periods of 15, 30 and 50 minutes at 37° C. were used. Samples were then held at room temperature and again read at the end of 2 and 20 hours from the start of the test. With herd samples, he found a progressive increase in positive reactions up to the 2-hour period with only about 1 percent increase occurring after 2 hours.

Standardization of Antigen Specificity

In spite of favorable reports concerning specificity of the ring test, there remained the problem of antigen standardization. Bruhn (1948) stated that examination of various lots of antigen prepared for use by the Danish State Veterinary Directorate, Copenhagen, Denmark, revealed much variation in antigen quality. By intensification of the staining procedure and by utilization of an acid wash of the stained antigen, workers at the Hygienic-Bacteriological Laboratory of the Royal Veterinary and Agricultural College of Copenhagen, Denmark, were able to produce a high grade standard antigen (Bruhn, 1948). The antigen was diluted with a glycerine-phenol solution, rather than the tap water previously used. According to Bruhn (1948), antigen pro-

duced by these improved methods made possible incubation of the ring test for 1 hour without risk of non-specific reactions.

These improvements and developments by the Danish and Swedish investigators are basically responsible for the present state of the ring test's sensitivity. As aptly pointed out by Blake *et al.* (1952), no major improvements have been made in the ring test in spite of the large amount of research that has been reported since the above mentioned period.

Introduction Into United States

The ring test was not applied to brucellosis control in America until 1947, when studies were initiated in Minnesota (Roepke, *et al.*, 1949) with a study of 30,811 herd ring tests. Encouraging results reported by these workers led to further field trials in other sections and the final adoption of the test by the U. S. Bureau of Animal Industry, as reported by Kuttler (1952).

Accuracy of the Ring Test on Pooled Milk

Agreement with the Blood Agglutination Test and Efficiency in Locating Herds. Following the report of Seit and Leth Jorgensen (1944) Christiansen (1948) published results of application of the ring test to milk samples from about 100,000 herds. Ring tests carried out simultaneously with blood tests on 6,266 herds showed complete agreement of 93.2 percent, partial disagreement in 2.4 percent, and complete disagreement in 4.4 percent of the cases. This compares favorably with the 96.2 percent overall agreement reported by Seit and Leth Jorgensen (1944). Combined results of these two groups of Danish workers show the ring test to be 82 percent efficient in locating infected herds. About two-thirds of the reactor herds failing to agree with the ring test were due to infected animals being out of production.

Roepke, *et al.* (1950), reported on a total of 30,811 ring tests of herds in 25 counties in Minnesota. These counties were on the area control plan. Herds were ring tested immediately preceding blood tests in as many cases as possible. Results show an overall agreement of 96.2 percent between the two tests. However, only 4.5 percent of the herds reacted positive to the blood test. Thus, as the authors pointed out, if the ring test had been negative throughout, an overall agreement of 95.5 percent would have existed. Based upon results of 8,469 herds, efficiency of the ring test to detect infected herds was found to be 68 percent. Of 107 failures to detect infected herds, 69 (64.5 percent) were due to the sole reactors in the herd being out of production. They found that the ring test detected 88 percent of the reactor herds where the infected animals were in production.

In a recent study, Morse, *et al.*, (1952b), reported an accuracy of 81.5 percent for the ring test in detecting herd milk from blood test positive producers. On blood test suspect herds, the ring test was positive in 14.1 percent instances and on blood test negative herds the authors report a ring test agreement of 94.8 percent. This gave an over-all agreement of 74.1 percent for the ring test with the blood test.

False Positive Reactions on Pooled Milk. The problem of false positive reactions or surplus reactions on negative herd milk was partially alleviated with improvements in antigen specificity. Literature shows some false positve reactions still occur on negative herd milk. Results of early investigators, Norell and Olson (1943), where presumably specific antigen was employed, indicated eight false positive ring tests out of a total of 225 herd samples, when compared with the blood agglutination test. This is an error of approximately 3.5 percent in surplus reactions for the ring test. Seit and Leth Jorgensen reported only 57 false positive ring tests out of 3,362 herd samples, or 1.7 percent Roepke, *et al.* (1950), found that only 11 percent of the herds with one plus ring test reactions were infected as indicated by the blood test. Two to four plus ring test reactions, however, showed 56 percent infected, 12 percent suspicious, and 32 percent negative when the herds were blood tested.

Results of a state-wide brucellosis control program in Wisconsin (Anon., 1952), involving some 130,121 herds, show a total of 53,915 of these testing positive to the ring test, or 41.4 percent. Of this number of reactor herds, 72.8 percent were found positive, 17.6 percent suspect, and 9.6 percent clean when blood tested. Thus, approximately 4.0 percent of the total herds ring tested were false positive when compared to the blood test.

Reasons for False Positive Reactions on Herd Milk. Information pertaining to reasons for the occurrence of false positive reactions on pooled milk is limited. Roepke, *et al.* (1950) reported freezing of negative milk frequently gave a false reaction of (+) or (++) intensity. These authors also have pointed out that when samples are taken from the dump vat there is a possibility of sample contamination from the previous lot of milk. Colostrum milk occasionally will impart a positive reaction to negative herd milk, even when diluted several times (Holm, *et al.*, (1950). These authors report that milk from cows infected with mastitis frequently give false positive results. However, due to the dilution effect, the reaction is never carried out higher than a 1:10 dilution. Age of the milk may be an influencing factor on ring test reaction. Bryan (1951) noted a more pronounced reaction in positive milk after three or four days of storage.

Sensitivity of Ring Test on Pooled Milk. Ability of the ring test to detect one reactor cow's milk when mixed with several lots of negative milk has been studied by several investigators. It has been shown to be reasonably accurate on pooled milk in detecting infected herds, even though positive milk represented only a small portion of the sample specimen. Norell and Olson (1943) indicate they were able to dilute milk from *Brucella* infected herds up to 40 times with negative milk without the ring test reaction disappearing. Bruhn (1948) demonstrated a positive reaction on milk from a positive cow diluted 256 times with negative milk. Huddleson (1949) reported detection of positive milk in dilutions of 1:40 to 1:160 with negative milk. Holm, *et al.* (1950) noted the detection of an infected cow's milk when mixed with that from 50 non-infected cows. Bryan (1951) stated that one ring test from a group of 25 to 50 cows will detect any animal shedding agglutinins in their milk. Morse, *et al.* (1952, 1952a), however, report their findings indicate that a cow reacting positive to the blood test at a titer of 1:100 to 1:200 would be missed in herd milk where one composite sample was taken from two or more cans for ring testing. On the other hand, they state that cows with a blood test reaction titer of 1:400 or above can be easily detected in herd milk under most conditions of sampling.

In some cases the ring test has been found more sensitive than the blood agglutination test for detecting early stages of infection. Roepke, *et al.* (1950) reported an investigation of 37 false positive herds by the ring test revealed that 6 were subsequently positive after an interval of 6 months. Bryan (1951) found that the ring test detects infection before the rise in blood titer. Likewise, Van Drimmelen (1951) noted that cows react partially to the ring test in the incubative stage of infection.

Accuracy of Ring Test on Individual Cow Samples

Agreement with the Blood Test and Efficiency in Locating Individual Infected Animals. Studies by various investigators suggest the accuracy of the ring test when performed on individual cow samples is somewhat below that obtained when herd samples are tested. Norell and Olson (1943) compared the ring test results on 453 individual cow samples with individual blood agglutination reactions. Of this group, 338 gave negative reactions with the ring test, of which 26 were positive to the blood test. The ring test was positive on 115 cows, of which 103 were positive to the blood test and one doubtful reaction. Thus, they were able to detect 103 infected cows out of a total of 129 reactors, or 79.8 percent. Twelve ring tests were false positive, a surplus of 9.3 percent of the 129 cases which were shown to be reactors

by the blood test. Bruhn (1944) reported that by examination of milk and blood samples from 587 cows, 123 reactors were found by the ring test, 97 of which reacted with a blood agglutination titer of 1:20 or higher. In the case of 464 cows testing negative to the ring test, 24 reacted positive to the blood agglutination test. This represents an 80.2 percent accuracy for the ring test in detecting individual infected animals. Approximately 5.3 percent of the total ring tests proved false positive. He found that the ring test failed in five instances where the blood titer was 1:100 or more.

Christiansen (1948), by simultaneous ring and blood tests upon almost 4,000 individual animals, found an 84.3 percent agreement, 4.1 percent partial disagreement, and a total disagreement of 11.6 percent when the ring test results were compared with the blood test. He found that in over 80 percent of the cases of disagreement, the ring test was positive and the blood test negative.

In more recent work, King (1951) reported results of a comparison of blood and ring tests on 428 individual cow samples. He found the ring test 96.3 percent efficient in locating infected animals, although a total of 16.3 percent false positive reactions were recorded. Bryan (1951) stated that his experiences from ring and blood testing of a large number of animals indicated the ring test to be only about 40 percent accurate when compared to blood serum reactions in clean or slightly infected herds. A breakdown of his results shows 129 animals positive to the ring test out of 8,750 ring tests performed. Of this number of ring test reactors, 51 had shown a blood test titer at some time, 47 had always been negative to the ring test, and 31 were disposed of for various reasons.

Effect of Vaccination upon the Ring Test Reaction

According to Hamilton and Hardy (1950), cows strongly positive by the blood test are almost invariably positive to the ring test and, conversely, those strongly positive by the ring test are almost always positive to the blood test. This is in agreement with Bryan (1951), who states that high blood titer animals are invariably positive in the ring test reaction. However, he states the reverse of this is not necessarily true. As expected, vaccinated animals gave a positive ring test where the blood test was positive. Holm, *et al.* (1950) found a majority of calfhood vaccinates gave ring test reactions similar to negative or suspect groups. Hamilton and Hardy (1950) report ring testing of milk from vaccinated cows gives confusing results and Manthei (1950) lists adult vaccination as one of the factors which may influence ring test results. Bryan (1951) states that he was unable to determine whether a blood titer was the result of infection or vaccination by use

of the ring test. Van Drimmelen (1951), however, holds views somewhat in disagreement with this. He claims that by a simple dilution technique he can differentiate between actively infected and vaccinated cattle with the ring test. His technique consists of placing the milk specimens in three test tubes at dilution rates of none, one-half, and one-fourth, using creamy milk from a negative cow as a diluent. Infected cows give positive results throughout dilutions whereas vaccinated cows do not. He states the test is applicable to cattle which have been vaccinated more than four months previously.

Inaccuracies in Ring Test When Performed on Individual Cow's Milk

False Positive Reactions. Christiansen (1948) noted that he found the greatest number of false positive ring test reactions when samples were from individual strippers, cows recently fresh, or those infected with mastitis. Holm, *et al.* (1950) likewise reported that mastitis, secretions from the udder of a dry cow, and colostrum were factors in false positive ring tests. They found that by employing a dilution technique, using fresh negative milk as a diluent, these factors could be overcome. A decolorizing factor was observed in colostrum milk which exaggerated the reading of slightly reactive samples. Huddleson also (1949) observed that colostrum milk when ring tested gave nonspecific reactions. However, he reported that cows with symptoms of mastitis gave no false positive reactions when the test was performed with antigen prepared by the technique he presented. Colostrum and abnormal milk were reported by Bryan (1951) to cause unreliable ring test results, and Hamilton and Hardy (1950) state that milk from cows infected with mastitis gave abnormal results which they interpreted as false positive.

Failure of the Ring Test to Detect Infected Cows. Several investigators have reported results which show that there are numerous exceptions to the statement that a strong reactor to the blood test almost invariably reacts strongly positive to the ring test (Hamilton and Hardy, 1950; Bryan, 1951). Bruhn (1948), in discussing the negative ring tests obtained upon five strong blood test reactor cows, concluded they were the result of lack of cream rising capacity because when negative milk was added to such samples, positive ring tests were observed.

In a study designed to investigate ring test accurracy, Holm, *et al.* (1950) observed an agglutination inhibition occurring on samples from animals with strong blood titers. They further noted this inhibition on the lower dilutions of pooled milk where the herd was heavily infected. The inhibition was most pronounced in samples from blood serum reactor animals that were nearly dry, dry, or producing colostrum. They

compared the condition to agglutinoid or prozone phenomenon. This work has been confirmed by Moore (1951), who observed the ring test failure on six out of 22 samples, although in each case the blood serum titer was 1:160 or higher. He attributed this inhibition to the prozone phenomenon. Boyd (1947) attributes this phenomenon to a specific inhibition of agglutination resulting from high serum concentrations.

Value in Brucellosis Control

Limitations of the Ring Test. The ring test has been shown to be much less reliable than the blood test when performed on milk from individual animals (Bruhn, 1948; Holm, *et al.*, 1950). It cannot detect infected animals which are not in production (Roepke, *et al.*, 1950). Milk from vaccinated animals, colostrum, and otherwise abnormal secretions have been reported to give false reactions even in some cases when from a pooled sample (Holm, *et al.*, 1950; Bryan, 1951). Freezing of samples also has been found to give false reactions (Roepke, *et al.*, 1950). All herds or individual animals reacting to the ring test should be blood tested before final disposition of animals is made, as the ring test is not a reliable diagnostic test in the strict sense (Roepke, 1951; Kuttler, 1952). Because of the above reasons, Roepke (1951) states that the ring test should be used only as a screening or presumptive test.

Advantages of the Ring Test. Cost of a county-wide ring test has been estimated by Roepke, *et al.* (1950) at about 10 percent of that for a county-wide blood test. As the authors stated, the ring test makes fewer blood tests required if applied rather frequently. At the same time it holds infection at a lower level than possible with blood tests alone at three year intervals. Many negative herds would not have to be bled. Some idea of the speed with which the ring test can be applied can be gained from the Minnesota project (Anon., 1949), which indicates that a staff of four veterinarians may average 1,000 to 1,200 herds per week. Roepke (1951) stated that the general advantages of the ring test are inversely proportional to the percentage of infection. He states: "In areas where the percent of infected herds is relatively high, that is between 15 and 30 percent of the herds, the value of the ring test is not as great. In such areas the blood test alone will serve to disclose one infected herd for each three to seven herds which are blood tested. In our counties under the area plan it is necessary to blood test from 50 to 200 herds to find one new center of infection. It is under the latter conditions that the ring test is particularly valuable. It enables us to locate a very high percentage of the infected herds without blood testing such large numbers of clean herds."

Modifications of the Ring Test

Centrifugation of Ring Tests. Holm, *et al.,* (1950 substituted a centrifugation technique in the ring test procedure in place of the incubation period. They observed that centrifugation of the tests at 1,500 r.p.m. for a period of 10 minutes gave more definite and clear-cut readings than were obtained with the usual procedure. They were also able to carry reactions out on positive samples to a greater dilution by centrifugation. Decolorization of antigen was prevented by reducing the time involved for bacterial growth and subsequent reducing action.

Ring Test for Cream. Necessity for cream testing arose during early stages of the Minnesota ring testing program as a fairly high percentage of Minnesota farmers market cream (Roepke, *et al.,* 1950). These investigators modified the ring test by adding to a small test tube 1 ml. of physiologic saline, two drops of a saturated sodium bicarbonate solution to neutralize any acid which might have been present in the cream, 12 drops of the cream specimen, and one drop of ring test antigen. Samples were agitated and allowed to stand at room temperature for 75 to 90 minutes before they were read in the same manner as milk samples.

The cream ring test results reported by Roepke, *et al.* (1950) showed the cream test's overall agreement with the blood test to be 70 percent, or 8 percent better than the 62 percent agreement for the milk tests. They stated that from the standpoint of sensitivity, the ring test on cream equalled a one plus greater reading on milk; e.g., a one plus cream reading appeared equivalent to a two plus reaction on milk, etc.

Capillary Ring Test. King (1951) introduced a modification of the ring test involving substitution of capillary tubes for the usual ring test tubes. He described the test as follows: Standard ring test antigen is drawn 5 to 7 mm. into a 90 mm. capillary tube of 0.8 mm. bore, followed by the milk sample to fill. Tubes are then inverted and the lower end placed in plasticene, allowing mixing of the milk and antigen. He states that strong reactor samples will show in five minutes and 95 percent of all reactions will be completed at the end of 15 minutes. Tests are read at the end of 30 minutes.

Advantages of the capillary test pointed out by King were saving in antigen, speed of completion, adaptation for homogenized and skim samples, and suitability for individual cow samples.

In a comparative study of the two tests, King (1951) reported an efficiency of 96.3 percent for the capillary ring test. However, the capillary ring test showed only 6.3 percent false positive reactions,

compared to 16.0 percent for the regular ring test on individual cow samples. Morse, *et. al.* (1952a) reported a closer agreement to the whey agglutination test with the capillary test than with the ring test on individual cow samples. In a later study, Morse, *et al.* (1952b) found an efficiency of 81.5 percent for the ring test and 77.6 percent for the capillary test in detecting blood test positive herds. On suspect herds, they obtained agreements of 14.1 percent and 9.2 percent for the ring and capillary tests, respectively, compared to the blood test. Their results indicate the capillary test gave 7.5 percent false positive reactions on clean herds, compared with 5.2 percent false positive for the ring test.

Milk Plate Modification of the Ring Test. A milk plate test for detection of brucellosis, employing ring test antigen, has been described by Blake, *et al.* (1952). Using standard plate test agglutination equipment, they placed 0.08 ml. of the milk sample on a glass plate with 0.03 ml. of ring test antigen and mixed. Optimum concentration of stained *Brucella abortus* cells in the antigen was found to be 3.0 percent. It was observed that a strong reaction occurred in a very short while. No significant changes in reactions occurred after 12 minutes. Readings were made on a basis of (−), (+), (++), (+++), and (++++), depending on the intensity of reaction.

The authors discovered the ring test would not detect agglutinins which had been added to goat milk. However, the plate method readily gave strong reactions on those samples containing added agglutinins. They reported they experienced no difficulty with the plate method in obtaining results on skim milk, homogenized milk, or milk containing as much as 40 percent fat. An efficiency of 99.7 percent in detecting infected cows was reported in their study.

Modification in Ring Test Antigen Production. Wood (1948) proposed a method for rapid production and standardization of antigen. His described method of staining and subsequent washing was quite similar to standard antigen procedure; however, the standardization technique he presented entailed serial dilution of stained antigen which was checked by ring tests upon negative and positive milks. Optimum antigen resulting from serial dilution tests was then tested on a positive milk sample which had previously been titrated by the slow whey agglutination method. The antigen was accepted as satisfactory if the last tube which showed a positive ring corresponded to the same dilution in the whey titration at which the end point was found.

Bendtsen (1949) describes a vital staining technique for the rapid production of ring test antigen. Living *Brucella abortus* strain 19 cells were washed from the agar surface with a solution of saline plus 1

percent glycerin. One gm. of 2, 3, 5 triphenyltetrazolium chloride was added to each 500 ml. of the concentrated antigen and the mass incubated at 37° C. for 20 hours. Antigen was ready for use after thinning with an equal part of the original diluent to which 1 percent phenol had been added. The author found that a comparison of this described antigen with the old ring test antigen gave remarkable agreement on 1,000 milk samples, about half of which were positive. Bendtsen points out the simplicity in preparation of this antigen, compared to standard procedure.

A similar technique for live staining with a tetrazolium salt has been described by Wood (1950). *Brucella abortus* cells were grown in the manner described by Brown and Wood (1948). To the suspension of living cells in broth was added an aqueous solution of 4,4'-bis(3,5-diphenyl-2-tetrazolinium) — biphenyl dichloride at a rate which gave a final concentration of one part in 16,000. The mixture was then incubated at 37° C. for four hours to permit organisms to reduce the compound and thereby become stained. Wood postulates that the reduction of the compound takes place inside the living cell. Thus, the antigenic specificity of the cell surface is not altered. After the incubation period, the mixture was heated for one hour at 60° C. to kill the cells. Killed stained cells were collected by centrifugation and resuspended to the desired density in 0.85 percent saline containing 0.5 percent phenol as a preservative.

The report stated that more than 100 different lots of antigen had been prepared during a two-year period using the described method, of which all lots were of uniform color intensity, specificity, and sensitivity. Ease in antigen production by this method was compared to standard procedure which, the author stated, is laborious and critical.

MATERIALS AND METHODS

Preparation of Ring Test Antigen

Cultural. A culture of *Brucella abortus* was secured from a vial of Strain 19 vaccine produced by Ashe Lockhart, Inc., Kansas City, Mo. The organisms were grown under increased carbon dioxide tension of 10 percent for the first five transfers; thereafter prolific growth occurred under normal conditions. Isolation of a smooth culture was accomplished by picking a smooth colony from a streak plate (Braun and Bonestell, 1947; Breed, *et al.*, 1948). The method of White and Wilson (1951) was employed occasionally as a check for smoothness. A medium of tryptose agar was used for both isolation and antigen cell preparation (Difco Lab., Inc., 1948).

For cell production the medium was modified to contain 2.5 percent agar for the purpose of adding rigidity to the medium. Agar solution

was poured into rectangular 32 ounce "Blake" bottles at a rate of about 75 ml. per bottle, sterilized, and allowed to solidify with bottles in a horizontal position. Inoculation was made by washing a smooth 36-hour culture into the bottle from a tryptose agar slant with 8 to 10 ml. of tryptose broth (Difco Lab., Inc., 1948). In some cases, the broth medium was inoculated with smooth culture and incubated for 24 hours before direct inoculation of the agar surface was made. The agar surface was completely moistened with inoculum, then incubated with the bottle in an upright position at 37° C. for a period of three to four days. This technique is a modification of that described by Castaneda (1947) for routine culturing of *Brucella*. Before harvesting, each culture was examined microscopically for purity and those showing contamination were discarded.

Cells were washed from the agar surface with a solution of 0.85 percent saline to which phenol had been added to give a final concentration of 1 percent. (This treatment with phenol was found to be ineffective in killing all cells. Agar streak plates revealed abundant *Brucella abortus* growth after four hours of contact. This fact has been previously reported by Huddleson, 1949). Cells were filtered through a finely meshed cotton cloth to remove extraneous matter and agar, then heated to 65° C. for 30 minutes (Huddleson, 1949). Following heat treatment, cells were collected by centrifugation in graduated centrifuge tubes at a speed of 2,600 to 2,800 r.p.m. for two hours. The supernatant was decanted and concentrated cells were resuspended in physiological saline adjusted to a pH of 4.0 for washing. They were then collected from this wash by the centrifugation treatment.

Staining. The staining solution and staining procedure were essentially the same as those described by Roepke, *et al.* (1949). Dilute oxidized hematoxylin stain was prepared as follows:

(a). Nine grams of ammonium sulfate was dissolved in 100 ml. distilled water, to which was added 30 ml. of glycerin.

(b). One gram of hematoxylin was dissolved in 2 ml. of ethyl alcohol (85 percent) by heating to 50° C. After solution was effected, distilled water was added to 100 ml.

(c). All of solutions a and b were mixed together and to this mixture 0.17 g. of sodium iodate, dissolved in 2 ml. of distilled water, was added. A period of 15 minutes was allowed for oxidation of the dye to take place, after which the solution was diluted 1:5 with a 10 percent solution of ammonium aluminum sulfate. Depth of staining of cells is dependent upon this rate of dilution. The diluted dye was aged for a period of 24 to 48 hours, then filtered through a wisp of cotton to remove insolubles.

Concentrated cells were added to the staining solution at a rate of 10 ml. of cell paste to 1,200 ml. of diluted stain. Suspension of cells in the staining solution was accomplished by homogenizing the mixture with a Fisher portable model homogenizer. The procedure of heating the mass to 65° C. and holding for three to five minutes before subsequent cooling and washing (Bruhn, 1948) was compared with the 12 to 20-hour holding period described by Roepke (1950). This latter staining procedure was employed routinely for all subsequent preparations to provide uniformity in depth of staining.

Collection of cells from the staining solution was accomplished by centrifugation at 2,600 to 2,800 r.p.m. for two hours. The concentrated stained cells were washed three times with physiological saline acidified with hydrochloric acid to a pH of 3.8 to 4.0. A fluid volume of one-fourth to one-fifth the original staining solution was used for each wash.

Stained cells were suspended in a solution of 0.5 percent phenol in a 50-50 mixture of glycerin and physiological saline at the rate of 1 g. of cells to 25 ml. of fluid. Suspension of cells in the final diluting fluid was accomplished on the first few lots by stirring the paste with a motor driven stirrer and gradually adding a few drops of glycerin-phenolized saline solution until a creamy suspension was obtained. The remainder of the diluting fluid was added and the mixture motor-stirred for a short period.

The practice of homogenizing the creamy suspension obtained by stirring a small amount of the diluting fluid into the stained washed cells with a subsequent homogenization of the entire mass was followed in the preparation of all but the first four lots of antigen.

Each lot of antigen was immediately compared with a standard antigen which reacted exactly with Bureau of Animal Industry ring test antigen. At least 20 samples, of which about half generally were from reactor cattle, comprised the material for antigen checks.

TESTING PROCEDURE

Sampling. Individual cow samples used in this study were obtained from the University dairy herd and two herds near Columbia. Herd samples were obtained through cooperation of two local dairies in Columbia. Field herd samples were taken from patron's cans as they arrived at the dairy intake. Individual cow samples were collected from the pail following milking of each cow. All animals in the University herd blood tested negative at a titer of 1:50. Samples from positive cows were secured from the two private herds and were from cows with a positive blood test or from recent Strain 19 vaccinates. Some positive herd samples were prepared by mixing together equal portions of milk from five cows, at least three of which represented samples

from blood test reactors or recent vaccinates. In each case, a well mixed sample of about 100 ml. volume was collected in a one-half pint milk bottle.

Quarter samples for study concerning the effect of mastitis on the ring test reaction were aseptically drawn. A split sample was tested for evidence of infection by means of the Hotis test and microscopic examinations (Merilan, *et al.*, 1950).

Bull semen samples were obtained from the University and the M.F.A. artificial breeding organization. Two samples of semen from a bull having a positive blood agglutination titer of complete at 1:100 were obtained from a private herd. These were the only semen samples obtained from positive animals. All bulls in the two previously mentioned herds blood tested negative at 1:50.

Ring Test Procedure

Age and Sample Temperature Study. Fresh warm samples were tested immediately upon arrival at the laboratory, before storing at refrigeration temperature of about 4.4° C. Samples then were ring tested at the end of each 24 hours for a period of three to four days. The ring test procedure, as described by Roepke, *et al.*, (1949) was followed essentially with some modifications. All samples were agitated thoroughly to redistribute fat in a uniform manner as it was thought that the amount of agitation received by an aged sample might be a factor in the ring test results. For uniformity in agitation, each sample was shaken 14 times in the manner prescribed for mixing samples preparatory to making standard plate count (American Public Health Association, 1948).

Duplicate 2-ml. test portions of the aged samples were poured into small test tubes (10 mm. x 75 mm.). Two drops of antigen were added to one unwarmed specimen. The other portion was warmed at 45° C. in a constant temperature water bath for a period of five minutes. The latter portion was taken from the water bath and allowed to cool momentarily at room temperature before adding antigen. Gum rubber stoppers were inserted into test tubes shortly after addition of the antigen. Samples were gently agitated to insure uniform mixing. Around 260 samples were refrigerated at one time. By the time these samples could be agitated and tests set up, the cold test portion had warmed from an average refrigeration temperature of about 4.4° C. to an average of 27° C. The 45° C. warmed duplicates had cooled to an average of about 32° C. Tubes were placed in racks in an inverted position to eliminate rundown of sample from the stopper and tube juncture to the fat column. All samples were incubated in a hot air incubator at 37° C. until the reaction was complete. This entailed a

period of one hour for the cold sample portion and two hours for the heated samples. The arbitrary standard for designating the degree of reaction as employed by earlier workers (Roepke, *et al.*, 1949) was used as follows:

— Cream line or ring white with the colored antigen in the skim milk fraction.

\+ Cream line approaching the color or equaling that of the skim milk fraction.

\++ Cream line slightly darker in color than the skim milk fraction.

\+++ Cream line significantly darker than that of the skim although the latter still contains appreciable color.

\++++ Cream line very dark blue with the skim milk fraction essentially white.

Fig. 1 illustrates positive and negative ring test readings.

Modification of Ring Test for Detection of Agglutinins in Body Fluids. Samples of semen were diluted at the rates of 1:25, 1:50, 1:100, and 1:200 using negative whole milk as a diluent. Controls were prepared with known negative semen at a dilution of 1:20, using the same milk dilutor as for the sample in question. Samples were ring tested as for milk without the heat treatment described above. A further control was run on the whole milk dilutor. This same procedure, only substituting physiological saline adjusted to approximately 4.0 percent fat content with fresh negative cream for a diluent, was employed for some samples.

Blood samples were tested in the same manner as semen, except for sample preparation. The specimen was allowed to clot and the serum was used as the test sample.

Test Procedure on Quarter Samples. The ring test procedure used for non-warmed samples was followed. Quarter samples were tested immediately upon their arrival at the laboratory before cooling.

Sample Preparation and Test Procedure for Frozen Samples. Fresh samples were ring tested on arrival at the laboratory. Specimens were subjected then to various freezing treatments which entailed alterations in time and temperature. This was accomplished by placing samples in the ring test tubes at the proper volume for ring testing. A centigrade thermometer was placed in a control tube and racks were placed in a cold chamber. An ice cream hardening room with controlled mean temperature of about -26° C. was used for this purpose. The fresh specimens were divided into split samples and both lots subjected to the freezing treatment. At the end of the treatment, both sets were allowed to thaw at room temperature. One group was ring tested with-

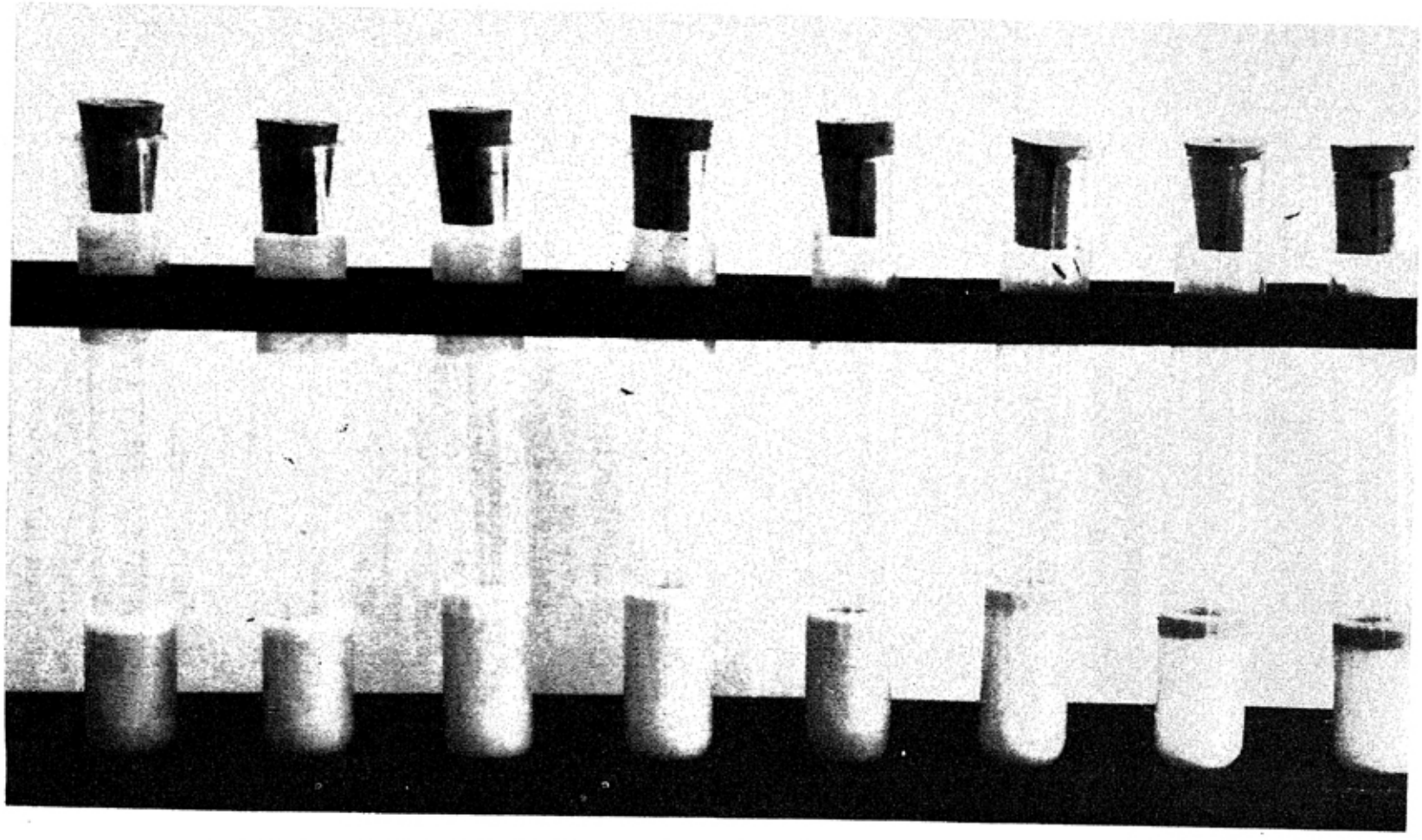


Figure 1 — Milk ring tests showing intensity of reactions. Left to right: Tubes 1, 2, and 3, negative; tube 4, +; tube 5, ++; tube 6, +++; tubes 7 and 8, ++++ reactions.

out further treatment. Temperature at the time of antigen addition was about 27° C. The duplicate frozen portions were subjected to the warming technique described for aged samples and the ring test procedure followed, as for whole milk, on both sets of samples.

Dilution Study Procedure. Dilution techniques reported by Holm, *et al.,* (1950) were employed, with some modifications. For a portion of the samples, fresh whole milk was used for dilution as described by previous investigators (Bruhn, 1948; Holm, 1950; Moore, 1951). However, distilled water, saline, tap water, and zeolite softened water were used as a diluent for some samples. After the samples were diluted, they were ring tested by the procedure used for whole milk.

RESULTS AND OBSERVATIONS

Antigen

All lots of antigen which were prepared according to the procedure described in Materials and Methods gave comparable results with a standard antigen. Several random samples of antigen were shipped to an impartial laboratory for comparisons with Bureau of Animal Industry ring test antigen. In each case the results were in complete agreement. These antigens were used as standards for prior and subsequent antigen comparisons. A total of 15 lots of antigen was prepared during the course of investigation. It was found that the homogenization technique as described in methods for suspending stained cells in the final antigen menstrum gave slightly more specific reactions than the non-homogenized product. Results of a typical antigen comparison are given in Table 1.

TABLE 1 -- ANTIGEN COMPARISON, A SELECTED NUMBER OF RING TEST REACTIONS*

Sample	Standard Antigen	Lot 10 Antigen	Lot 10 Homogenized Antigen
1	++++	++++	+++
2	++	++	++
3	++	++	++
4	++++	++++	++++
5	+++	+++	+++
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	+	+	-

*Individual milk samples from positive, recent adult vaccinated and negative cows.

Several lots of experimental antigen were prepared by modifying the culturing procedure from that described in Materials and Methods. One lot of antigen was prepared in the regular manner with the ex-

ception that a rough culture was selected for inoculation of agar bottles. Microscopic examination for purity revealed no contamination. Resulting antigen from this rough culture resembled the regular product in depth of stain but antigen comparisons revealed a lack of sensitivity. Ring test positive milk samples gave cream lines practically free of color when tested with this antigen. This may be the reason earlier workers were forced to use a 20 percent cell concentration rather than the four percent now employed (Bruhn, 1948).

Another modification entailed the addition of 2 percent sodium acetate (McIlroy, *et al.*, 1948) to tryptose broth medium, which was employed in washing cell growth from the agar slant tube to the bottle agar surface for inoculation. The same precedure was followed after this point as described for antigen production in Materials and Methods. Comparisons showed this antigen to be much more sensitive than the standard test antigen and consequently a much greater degree of non-specificity on negative individual cow samples was found. Table 2

TABLE 2 -- A SELECTED NUMBER OF NEGATIVE SAMPLES SHOWING THE DEGREE OF NON SPECIFICITY OF ACETATE GROWN ANTIGEN

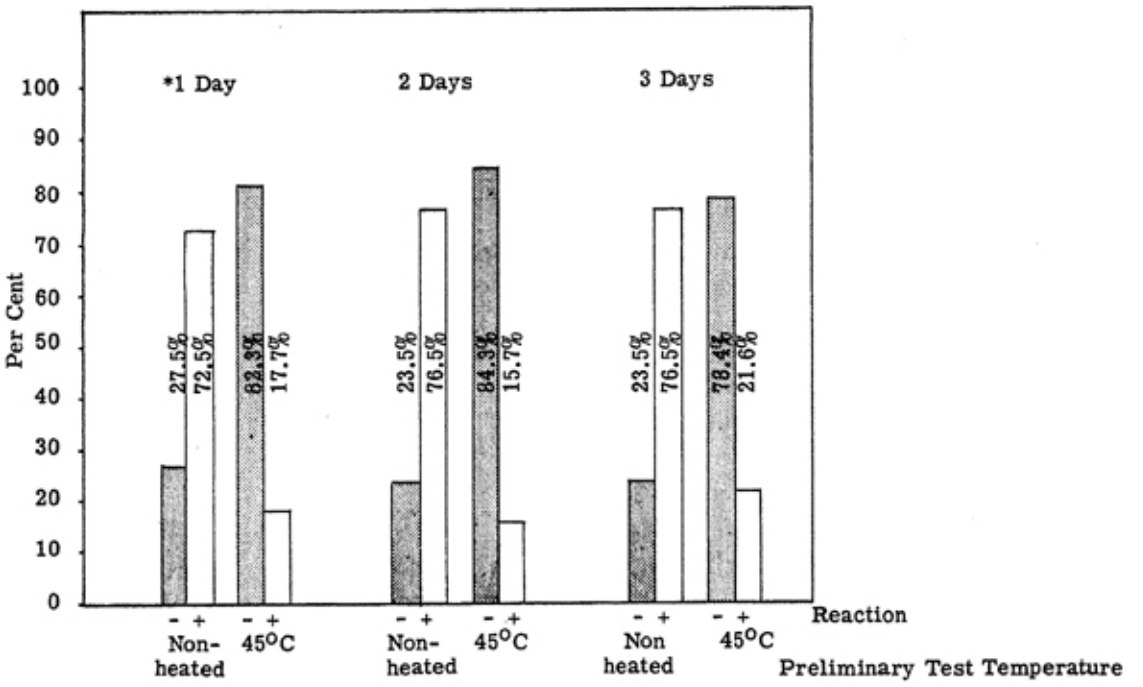
	Sample														
Antigen	1	2	3	3	5	6	7	8	9	10	11	12	13	14	15
Standard Antigen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetate Grown Antigen	++	+	-	+	++	++	+++	++	+	+	-	-	-	-	-

Individual fresh milk samples from blood test negative cows.

shows typical reactions of this non-specific antigen when compared to standard antigen. The *Brucella abortus* colonies from this preparation appeared to be smooth. During examination for purity, varying degrees of viscosity were revealed in some of the preparations.

A total of 404 samples was ring tested, employing this non-specific antigen. In this study samples were treated as in the age and preliminary heat treatment study, with the exception that the first ring tests were made after one day of storage under refrigeration. Combined results of the two ring testing procedures, comprising a total of 1,847 ring tests, are shown in Figure 2. Percentage figures in the graph show only total positive reactions, which include all degrees of ring intensities.

A breakdown for the non-heated ring test portion shows 33 percent (++++), 22 percent (+++), 10 percent (++), 13 percent (+), and the remainder negative at the end of one day of storage. The (++++) and (+++) reactions increased 9.8 percent and 7.9 percent, respectively, for the three-day period. The preliminary heat technique of 45° C. gave slightly more of the doubtful reactions



*Milk Aged under Refrigeration Temp. of 4.4°C. For days Indicated

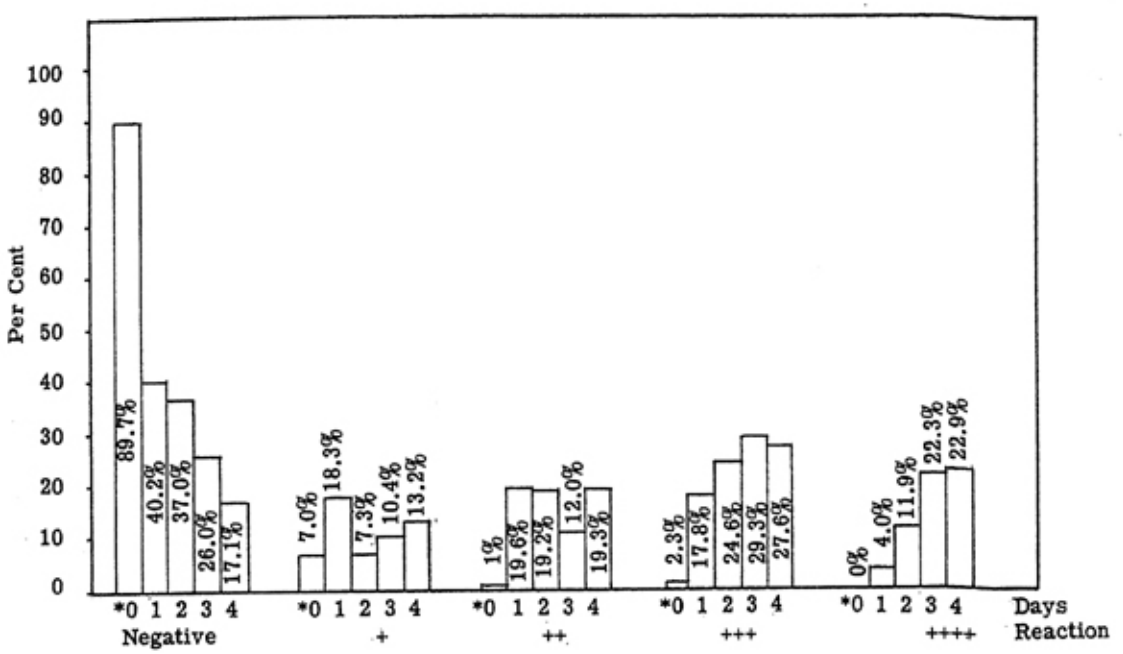
Figure 2 — The effect of preliminary sample warming upon ring test reactions where non-specific antigen was employed.

than the stronger (+++) and (++++) readings for the first day of storage.

There was only a slight trend toward more positive readings during the three-day period. It is interesting to note close comparisons between pre-heat treated sample results shown in Fig. 2 and "standard" antigen results from the same sample treatment, shown in Fig. 4. Agreement apparently is due to elimination of the Schern-Gorlish reaction by sample heat treatment where non-specific antigen was employed.

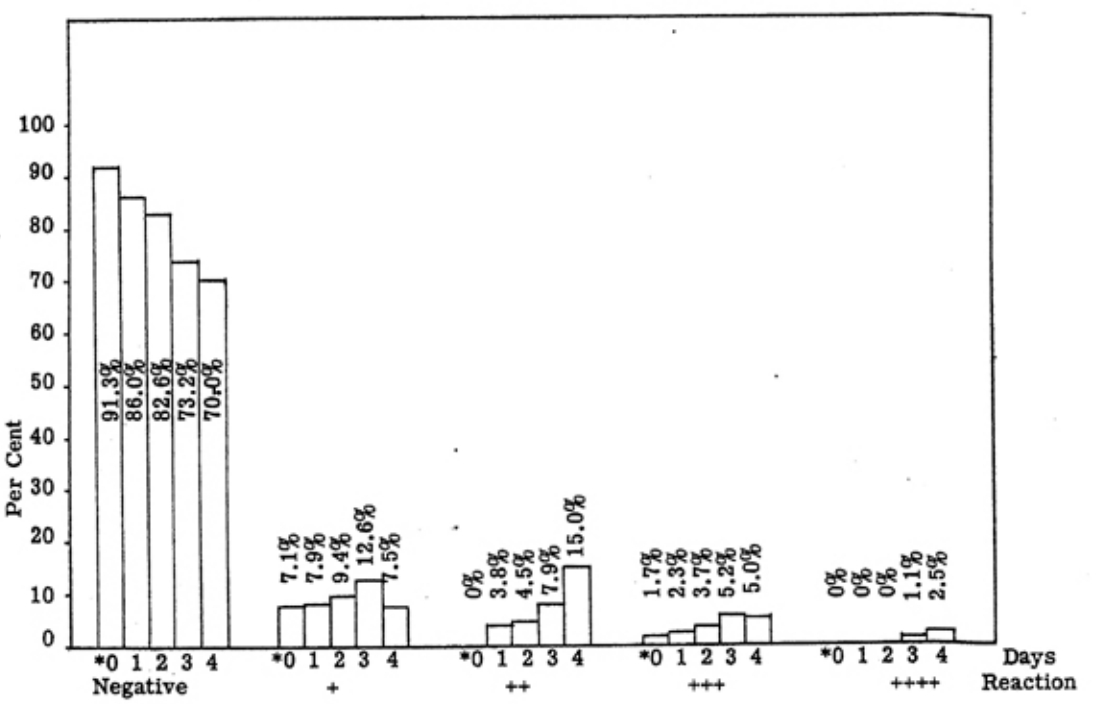
Effect of Age and Test Temperature of Milk

Effect of age upon the ring test reaction of 217 samples of individual cow's milk comprising 1,292 tests is shown in Figures 3 and 4. All samples were from blood test negative cattle at a titer of 1:50. Results of tests conducted in the standard manner without preliminary warming are illustrated in Fig. 3 on a percentage basis. It is interesting to note the large reduction in negative ring tests brought about by only 24 hours of storage under refrigeration. This trend continues throughout the duration of the holding period with only 17.1 percent of the samples remaining negative at the end of four days, compared to 89.7 percent negative for the fresh warm samples. The large initial decrease of 49.5 percent in negative reactions, which occurred



Samples from blood test negative cows refrigerated at 4.4°C.
*0 days indicates reaction of fresh warm milk.

Figure 3 — Effect of age on ring test reaction of milk when tested without preliminary warming.



Samples from blood-test Negative cows refrigerated at 4.4°C
*0 Days indicates reaction of fresh warm milk.

Figure 4 — Effect of preliminary warming at 45° C. for 5 minutes on ring test reaction of aged milk.

in the first 25 hours, is largely the result of increased (+) and(++) reactions; however, some negative samples increased to (+++) reactions and a few to (++++) readings.

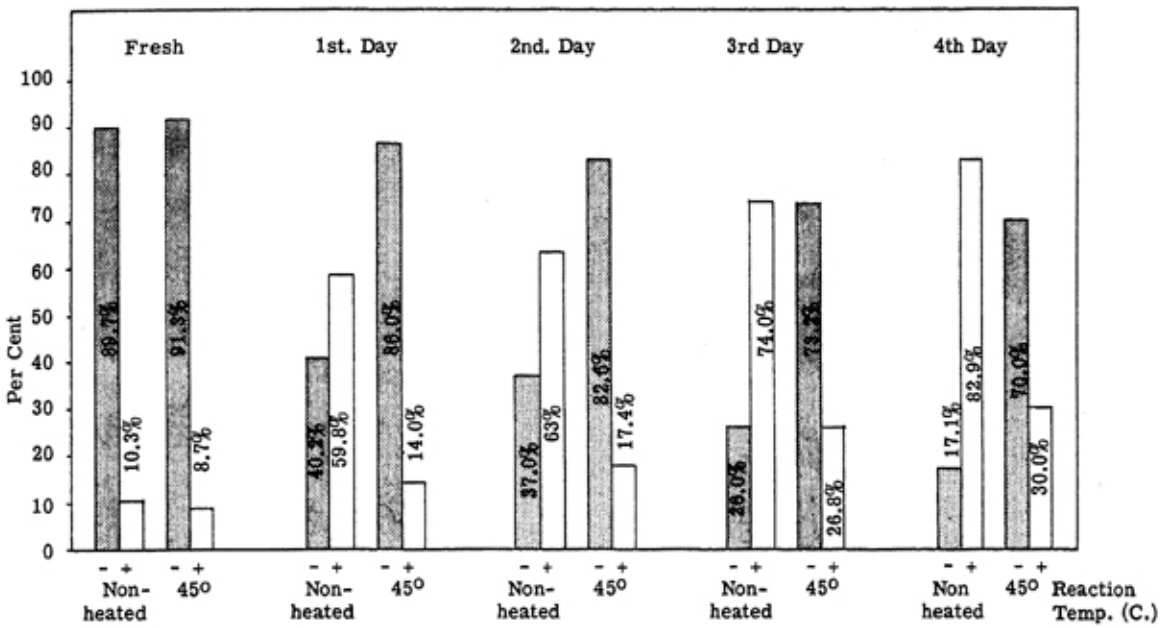
As indicated by the graph, there was a steady increase of (+++) and (++++) readings up to the fourth day. These steady increases were predominantly the result of a change in the previous day's (+) and (++) readings.

Figure 4 shows the effect of preliminary heat treatment of 45° C. upon duplicate samples from the same source as those presented in Figure 3. In arriving at the 45° C. temperature for preliminary sample treatment, a series of pilot tests was run on negative milk at the following temperatures: 30°, 37°, 45° and 50° C. False positive reactions induced by age decreased as the temperature increased to 45° C. With an increase in temperature above this figure there was no change in reaction and it was accepted as the test temperature. It was observed that all preliminary heat treatment of samples above 37° C brought about a progressive increase in time required for cream line formation.

At least one and one-half hours were required and two hours were accepted as the incubation time. Some antigen decolorization was noted from bacterial action on low grade milks during this increased incubation period. Two drops of a saturated sodium bicarbonate solution were added in some cases to overcome this (Roepke, *et al.*, 1948). There was a definite increase in positive reactions with each successive day's ageing using the warming treatment prior to testing. However, the figures show only slight increases for each 24-hour period. This reduction in negative readings, moreover, was predominantly a change to the more doubtful reactions of (+) and (++) intensity.

A composite summary of Figures 3 and 4 is shown by Figure 5. A comparison of the standard testing procedure with the modified prewarming technique is illustrated using a total of the positive and negative reactions for percentage figures. Reactions of the warmed 24-hour portions compare favorably with those of the fresh non-heated samples, particularly when the number of weakly positive reactions for that series is considered.

As shown by the graphic comparison, slightly more of the heat treated samples when tested fresh were negative than those represented by the fresh non-heated reactions. However, as the number of tests indicates when compared to the total sample number, not all samples were tested when fresh and a few were not held for the entire four-day period. Thus, some slight percentage differences may be attributed to this factor.



Samples Aged under Refrigeration Temp. of 4.4°C. For Days Indicated

Figure 5 — Summary of ring test reactions showing a comparison of preliminary warming procedure with standard ring testing technique.

A study of the effect of preliminary heat treatment upon positive samples was made using 45 herd samples and 46 samples from individual reactor or adult recently vaccinated cows. A total of 90 ring tests were performed upon herd samples, representing duplicate specimens of the milk; one set tested by the standard precedure and the other employing the modified warming technique. Results of these tests on herd samples are shown in Table 3, which compares the amount of

TABLE 3 -- A COMPARISON OF PRELIMINARY HEAT TREATMENT WITH STANDARD TECHNIQUE UPON THE RING TEST REACTIONS OF 45 REACTOR HERD SAMPLES

Ring Test Reaction Intensity	Non-heated	Preliminary Heat of 45° C. for 5 Minutes	Reading Changed by Heat Treatment	
	No. of Samples	No. of Samples	Increase	Decrease
+	0	0	0	0
++	4	8	0	0
+++	18	13	2 to (++++)	*4 to (++)
++++	23	24		*1 to (+++)
Totals	45	45	2	5

*Samples taken at intake with no differentiation of morning and night milk.

change brought about by warming procedure against the standard method of testing. As shown in Table 3, five of the samples decreased in ring intensity when milk was warmed. In every case, these reductions in readings represented samples taken at the plant receiving platform

where no differentiation between night and morning milk was made. The two increases in reaction apparently brought about by the heat treatment were from tests performed on fresh warm milk. No changes greater than a (+) reading were observed.

Data in Table 4 indicate the effect of heat upon fresh warm milk from positive cows. A total of 92 ring tests were conducted on 46 individual cow samples. Eleven reductions were noted in ring intensity; ten of which were of a (+) intensity with one representing a (++) decrease. This reduction in ring intensity could not be accounted for as in the herd milk phase. However, when data from the dilution study were examined, it was observed that the prozone phenomenon was intensified by heating. Three out of four of the decreases can be attributed to this factor. None of the other seven were included in the dilution study.

TABLE 4 -- A COMPARISON OF PRELIMINARY HEAT TREATMENT WITH STANDARD TECHNIQUE UPON THE RING TEST REACTIONS OF 46 POSITIVE COW SAMPLES

Ring Test Reaction Intensity	Non-heated	Preliminary Heat of 45° C. For 5 Minutes	Readings Changed by Heat Treatment	
	No. of Samples	No. of Samples	Increase	Decrease
+	2	5		
++	8	9	1 to (+++)	3 to (+) 1 to Neg.
+++	17	13		*6 to (++)
++++	19	18		1 to (+++)
Total	46	45		
		(1 negative reaction)		

*Three reductions attributed to intensification of prozone phenomenon by warming.

Effect of Freezing and Heat Treatment Upon Ring Test Reaction

A total of 115 samples resulting in 455 ring tests comprised the study to determine effect of freezing and subsequent preliminary sample warming of 45° C. upon the ring test reaction. Milk samples were treated in the following ways for study: One duplicate set was chilled to -5° C., then removed and placed at room temperature for thawing; another set was chilled to -20° C. before removal for thawing; and a third set was frozen at -15° C. for 16 hours before thawing. Samples were also ring tested after 48 hours of storage at 4.4° C.

It was observed that freezing of negative samples created false positive reaction, which is in agreement with Roepke, *et al.*, (1950). False positive reactions increased both in number and intensity with increasing freezing treatment. Results of a representative group of

sample reactions are shown in Table 5. It is interesting to note the similarity found in ring test reactions of duplicate milk samples, one of which had been frozen to -20° C. and the other stored under refrigeration for 48 hours. The 45° C. pre-warming technique was found to decrease false positive reactions at about the same rate as for aged samples.

TABLE 5 -- THE COMPARATIVE EFFECT OF FREEZING AND AGEING OF MILK SAMPLES RING TESTED BY PRE-WARMING AND NON-WARMING TEST PROCEDURE

Sample	Fresh Milk	*Frozen to -5° C. Thawed to 27° C.	*Frozen to -5° C. Pre-warm at 45° C.	*Frozen to -20° C. Thawed to 27° C.	*Frozen to -20° C. Pre-warm at 45° C.	Stored at 4° C. 48 Hours Non-heated	Stored at 4° C. 48 Hours Pre-warm at 45° C.
1	-	-	-	-	-	-	-
2	-	++	-	+++	-	++	-
3	-	+	-	++	-	+++	-
4	-	++	-	+++	-	++	+
5	-	++	-	+++	-	+++	-
6	+	+++	++	+++	++	+++	+++
7	-	-	-	+++	-	++	-
8	-	-	-	++	-	++	-
9	-	+	-	+++	-	+++	-
10	-	+++	++	+++	++	+++	+++
11	-	-	-	+	-	+++	-
12	-	-	-	+	-	+	-
13	-	-	-	+	-	++	-
14	-	+	-	++	-	++	-
15	-	-	-	-	-	-	-

*Fresh samples chilled to desired temperature, then withdrawn and placed at room temperature for thawing.

Modification of Ring Test for Detection of Agglutination in Body Fluids

Ring tests were conducted on 73 samples of semen obtained from about 50 bulls, a total of 280 tests being made. All samples from negative blood test bulls gave negative ring test reactions. Two samples from one bull having a blood agglutination titer of complete at 1:100 gave positive ring test reactions throughout the dilution range of 1:25, 1:50, 1:100, and 1:200. No greater dilutions than 1:200 were employed. Reactions for the dilutions as indicated above were read as (++++), (++++), (+++) and (++), respectively. Figure 6 shows pictures of the ring test dilution series for the positive semen samples taken at the end of the incubation period. Ring test results of a typical negative semen sample are illustrated in Figure 7.

It was noted that introduction of semen to the milk diluent brought about wide, loosely packed cream rings, particularly in the lower dilutions. Occasionally incorporation of the skim milk fraction into this wide cream line gave a mottled, false positive reading with negative

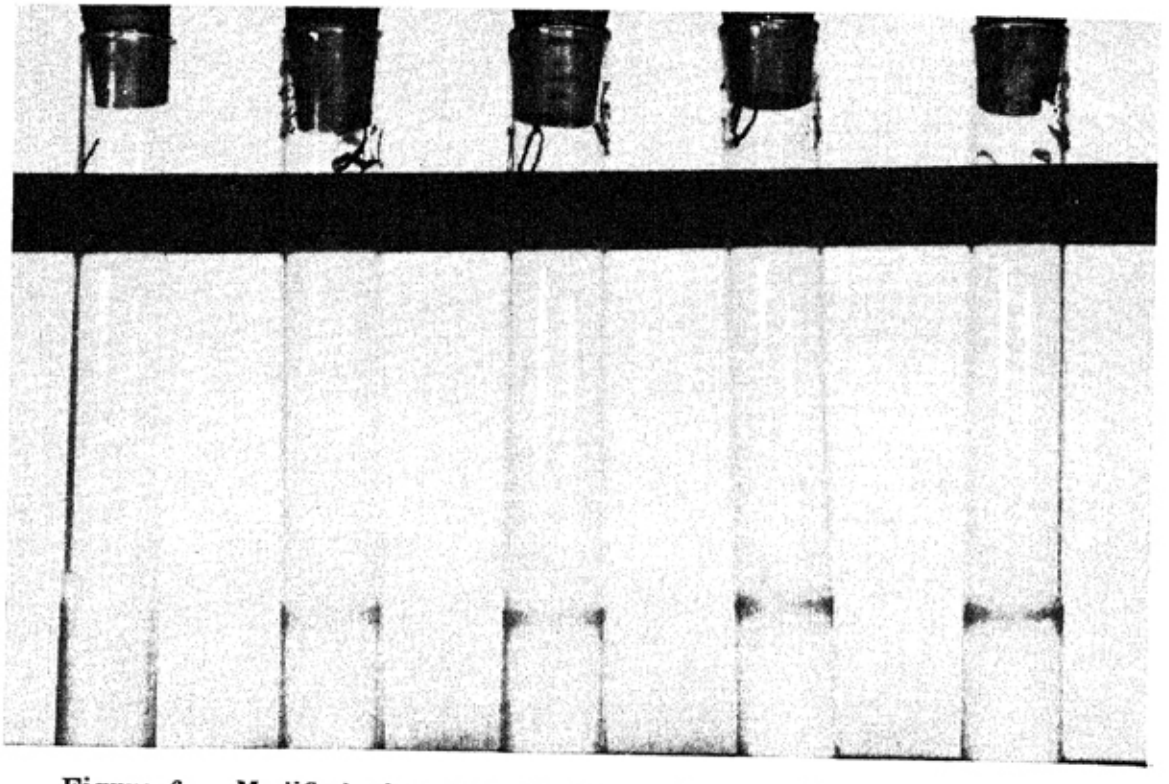


Figure 6 — Modified ring test on semen from a blood test positive bull. Left to right: Tube 1, negative whole milk diluter; tubes 2, 3, 4, and 5 represent dilutions of 1:200, 1:100, 1:50, 1:25 semen to milk, respectively.

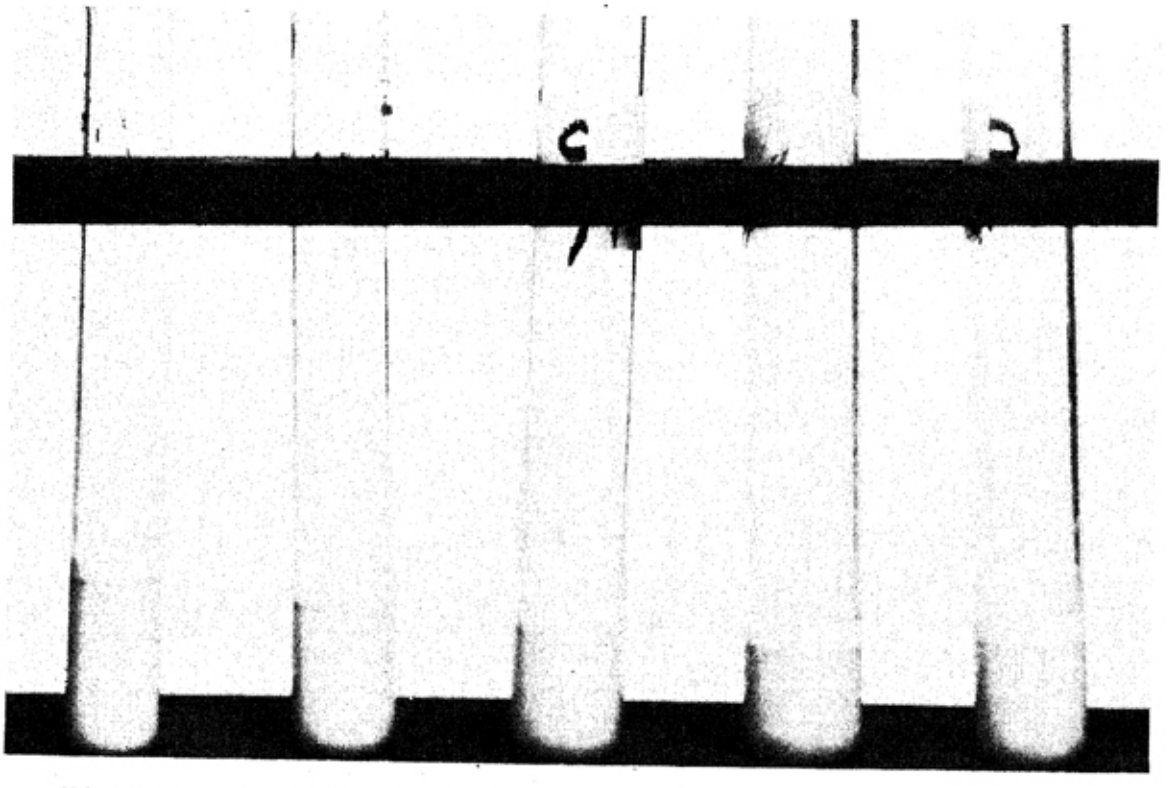


Figure 7 — Modified ring test on semen from a blood test negative bull. Left to right: Tube 1, negative whole milk diluter; tubes 2, 3, 4, and 5 represent dilutions of 1:200, 1:100, 1:50, 1:25 semen to milk, respectively.

samples. However, an increase of the incubation period to one and one-half hours partially eliminated this problem. Centrifugation of semen samples, using the resulting supernatant as a sample specimen, has further improved the procedure, although results of this technique performed on positive semen samples are not available. The procedure of substituting centrifugation for the incubation period as described by Holm, *et al.*, (1950) gave good results performed on negative samples. No positive semen samples have been tested with this technique.

Several samples of diluted semen were tested by adding negative cream to specimens at the rate of 4 percent. Samples were ring tested then as for milk. All samples reacted positive, including the controls. Subsequent investigation revealed that the combination of sodium citrate and egg yolk found in the egg yolk-citrate diluter (Herman and Ragsdale, 1950) was responsible for the false reaction, whereas neither alone with the cream added would cause it. Addition of fresh semen to the citrate diluter without egg yolk also gave a positive reaction when ring tested using negative cream as above. By addition of calcium ion in the form of $Ca\ Cl_2$ or $Ca(NO_3)_2$ to the egg yolk-citrate diluter, this false positive reaction was overcome. As no semen from a reactor bull was tested in this manner, it is not known what the ring test results would be with a combination of calcium, egg yolk-citrate diluter, positive semen and cream.

A total of 48 blood samples were ring tested at dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400 using negative herd milk as a diluent. All blood sample specimens having a negative blood agglutination titer of 1:50 gave negative ring test results. Table 6 shows typical ring test re-

TABLE 6 -- RING TEST AND BLOOD AGGLUTINATION TEST REACTIONS OF EIGHT REPRESENTATIVE BLOOD SPECIMENS

	Ring Test Reaction						Reactions to Blood Test			
Sample	1:25	1:50	1:100	1:200	1:400	Controls	1:50	1:100	1:200	1:400
1	-	-	-	-	-		-	-	-	-
2	+++	++	-	-	-		+	I	-	-
3	++++	+++	++	-	-		+	+	-	-
4	+++	++	-	-	-		+	+	I	-
5	+++	++	-	-	-		+	+	-	-
6	++++	++++	+++	+	-		+	+	+	+
7	+++	+++	+	-	-		+	+	-	-
8	+++	++	+	-	-		+	+	-	-
Negative Blood Milk Diluter	-					-				

sults of eight blood samples with blood agglutination titers ranging from negative at 1:50 to positive at 1:400. A comparison of the reaction titer of the two tests indicates the ring test modified procedure is slightly

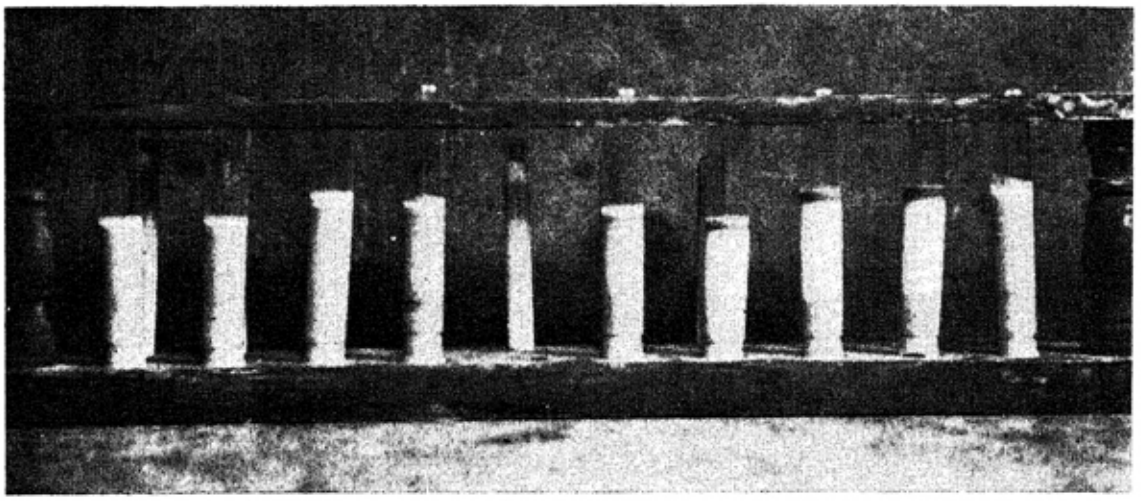


Figure 8 — Modified ring tests on blood serums from positive and negative samples. Left to right: Tubes 1, 2, 3, and 4 represent dilutions of 1:200, 1:100, 1:50, 1:25 negative blood serum to milk; tubes 5, 6, 7, and 8 represent dilutions of 1:200, 1:100, 1:50, 1:25 positive blood serum to milk; tube 9 represents negative milk dilution.

less sensitive than the blood test in these studies. Reactions of a typical ring test on a positive blood specimen using herd milk for a diluter are illustrated in Figure 8.

Centrifugation, in place of the incubation technique for ring formation, was tried for ring testing of blood. This technique gave a clear positive ring test when positive blood samples were tested at about the same intensity as for the incubation method. There was less layering of the cream ring than was encountered with the one-hour incubation technique, although with the latter procedure this was not significant even at the smallest dilution of 1:25. A centrifugation speed of 1500 r.p.m. was found to give false negative results and a speed of 600 to 1100 r.p.m. for 12 minutes was employed for best results.

A centrifuge head, 16 inches in diameter was used. Eight positive blood serum specimens were ring tested using negative goat milk in place of cows' milk for a diluter. It was found that the results were positive with a ring test reading of (+++) at 1:25 blood to milk dilutions where the blood agglutination titer was positive at 1:50 or higher. Blood to goat milk dilutions of 1:50 or higher gave negative or, in some cases, doubtful ring test readings. The serial dilution ring test reactions on these same eight samples where cows' milk was used as the diluent are shown in Table 6. Tests were performed by the standard ring test incubation procedure because it was found that centrifugaton, in place of incubation, with goat milk gave results quite variable toward the negative reading in comparison. It was interesting to note agglutination on some of the goat milk samples at higher dilutions could

be observed on the side of the ring test tube after several hours of incubation, even when the cream ring was negative.

By a using 1:1 or 1:2 cow to goat milk dilution, reaction titers on positive blood serum similar to cows' milk alone were obtained.

Ring Test Comparisons with Laboratory Diagnostic Tests for Mastitis

A total of 265 quarter samples were simultaneously ring tested and laboratory tested for evidence of mastitis. This number of quarter samples represented the total quarters in production from 67 blood test negative cows. A ring test also was run on a composite sample from the individual cow milking. All samples were from blood test negative cows.

No apparent relationship was found to exist between milk samples positive to both the mastitis tests and the ring test. It was noted that a few milk samples showing gross clinical evidence of mastitis could not be read due to lack of cream rising capacity. A percentage summary of results revealed 19.6 percent of the quarter samples positive for mastitis and 16.6 percent positive to the ring test. Individual total cow samples were 14.8 percent positive to the ring test. Table 7. shows

TABLE 7 -- A COMPARISON OF RING TEST REACTIONS WITH LABORATORY DIAGNOSTIC TESTS FOR EVIDENCE OF MASTITIS

	Ring Test Reaction Quarter Samples				Ring Test Reaction	Laboratory Diagnosis for Mastitis Quarter Samples			
Cow	Left Front	Left Rear	Right Front	Right Rear	Combined Milk	Left Front	Left Rear	Right Front	Right Rear
3	-	-	-	-	++	-	-	-	-
13	++	-	-	-	-	-	-	-	-
15	-	++	-	++	-	+	-	+	-
16	-	-	+++	+++	++	-	-	-	-
17	+++	+++	Blank	++++	++++	-	-	Blank	-
25	++	+	++	++	+	-	-	-	-
27	-	-	-	-	+++	+	+	+	+
29	+	++	+++	++	+++	-	-	-	-
31	+++	++	+++	+++	-	-	-	-	-
32	+++	+++	+++	+++	-	-	-	-	-
34	+++	+++	+++	+++	++++	+	+	+	+
42	-	++	++	-	++	-	-	-	-
44	+++	++	++	-	-	+	-	-	-
46	++	-	-	-	+	+	+	+	+
48	++	-	-	-	-	-	-	-	-
50	++	++	+	+	-	-	-	-	-
55	-	++	-	++	-	-	-	-	+
62	+	+++	-	-	-	-	-	-	-
66	-	-	-	-	++++	-	-	-	-
67	++	-	-	-	-	+	+	+	+

(47 sample groups entirely negative to the ring test are excluded)

Above results of tests completed on samples from 67 blood test negative cows.

a condensation of results from this study with all samples excluded which were negative to the ring test.

It is interesting to note in the table that samples from cows 3, 27, and 66 showed negative quarter foremilk ring test whereas the milk samples for the whole milk gave a positive reaction. On the other hand, samples from cows 13, 15, 31, 32, 44, 48, 50, 62, and 67 showed from one to four positive quarter reactions with resulting negative entire milk ring tests.

Effect of Diluters, Dissolved Salts, and pH on Ring Test Reaction of Milk

Diluters. A total of 159 milk samples from individual cows were included in the dilution study, which comprised 493 ring tests. Samples were diluted with fresh warm milk, physiological saline, tap water, and zeolite softened water at rates of 1:1 to 1:5 milk specimen to test material. Not all of the milk samples were included with each diluter study.

It was observed that dilutions of 1:1 through 1:5 specimen to herd milk gave best overall results in that problems of prozone phenomenon and inadequate creaming were eliminated. Results with physiological saline as a diluent closely approximated those for negative herd milk, with the exception that specimens with poor cream rising capacity were not particularly aided by dilution. However, cream lines formed quicker and were larger and clearer with the saline dilution, in comparison to whole milk ring test. Prozone phenomenon was eliminated, as in the case of negative herd milk diluter. Table 8 shows a comparison of

TABLE 8 -- A REPRESENTATIVE NUMBER OF RING TEST REACTIONS SHOWING THE EFFECT OF DILUTION ON THE RING TEST REACTION
(Individual Milk Samples From Recent Vaccinates and Positive Cows)

Sample	Fresh Milk	1:1 Sample to 0.85% Saline	1:1 Sample to Neg. Milk
1	++++	++++	++++
2	++	+++	++
3	-	++	+++
4	++	++	+++
5	*N. C.	*N. C.	++
6	++++	++++	++++
7	+++	++	+++
8	-	+++	+++
9	-	++	+++
10	++	++	+++

*N. C. = No Cream Line.

ring test reactions on milk samples diluted with negative herd milk and physiological saline. Three samples (numbers 3, 8 and 9) which gave ring test reactions interpreted as showing evidence of prozone phenomenon on undiluted milk are included in the selected group.

A group of 23 positive individual cow samples were diluted with physiological saline at the rate of 1:1. Ring tests were performed on the undiluted and diluted portions, using both regular and preliminary warming ring test techniques. A representative group of ring test reactions from these milk samples are shown in Table 9. It is interesting

TABLE 9 -- A COMPARISON OF NON-HEAT TREATMENT WITH PRELIMINARY HEAT TREATMENT ON DILUTED AND UNDILUTED FRESH MILK SAMPLES
A Representative Number of Samples from 23 Blood Test Positive and Recent Vaccinate Cows

	Ring Test Reaction			
	Non-heat Treated		Pre-heat at 45° C.	
Cow	Undiluted Milk	1:1 Dilution 0.85% Saline	Undiluted Milk Pre-heat at 45° C.	1:1 Dilution 0.85% Saline
1	+++	++++	+++	++++
2	+++	++++	++	++++
3	+++	++++	+	++++
4	*++	++++	++	++++
5	++++	++++	++++	++++
6	++	+	+	+
7	+++	++++	+++	++++
8	-	+	-	+
9	*-	++++	*+	++++
10	+	++++	+	++++
11	+++	+++	+	++++
12	-	+++	-	+++
13	+++	++++	+	++++
14	*-	+++	+	+++
15	++++	++++	++++	++++

*Poor creaming ability

to note the decided reduction in ring test reactivity on pre-heated, undiluted samples, compared to the non-heated test portions and pre-warmed diluted portion. The zonal phenomenon also was interpreted responsible for reduction in ring intensity of samples 8, 10, and 12, when tested undiluted and non-warmed. In samples 4 and 14, the saline dilution was noted to improve cream rising capacity to the extent that clear ring test readings were demonstrated.

Several samples of fresh, positive herd milk were diluted at rates of 1:1, 1:2, 1:3, 1:4, and 1:5 milk to physiological saline. Cream lines of these pooled samples were thin at the 1:5 dilutions, but readings could still be obtained. Results indicate water dilutions of greater than 1:4 with herd milk and 1:2 with individual cow samples gave scanty cream lines and questionable readings on some samples.

Dilution with cold tap water gave results comparable to those obtained with physiological saline. However, it was found that false positive ring reactions were induced to a great degree when fresh milk from individual negative cows was diluted with zeolite softened tap water.

Apparently, the amount of zeolite present in the water was sufficient to bring about a non-specific agglutination of the antigen.

Dissolved Salts. Investigation of the effect of sodium citrate upon ring test reaction in the bull semen study was expanded to include sodium oxalate, disodium phosphate and sodium tartrate. Preliminary investigations revealed that only the sodium oxalate of the three latter compounds had any influence upon ring test reaction when added to milk containing fresh semen. Salts were added to milk containing fresh semen at a rate calculated to give a final molar concentration of 0.07, which is approximately the concentration of sodium citrate in the egg yolk-citrate diluter.

Addition of sodium citrate and sodium oxalate to milk gave more false positive ring test reactions than were obtained on the untreated milk portion. Addition of fresh semen, egg yolk, or egg white at a dilution of 1:25 serum to milk, to these samples containing sodium citrate or sodium oxalate further increased the false positive ring test results. Results were not consistent in all cases, particularly where the salt concentrations were lowered. It was found that additions of calcium chloride or aluminum chloride at the same molar concentration as sodium citrate or sodium oxalate in the milk eliminated false positive reactions.

Addition of two drops of a saturated sodium bicarbonate solution (Roepke, *et al.*, 1950) to 2 ml. of milk gave ring test results slightly less sensitive than those obtained on untreated samples. This was predominantly a change in the doubtful reading of (+) to negative. A total of 448 milk samples were tested by this technique and compared to the non-treated reactions. One lot of antigen was prepared by suspending stained cells in a saturated solution of sodium bicarbonate. No differences in reactions with this antigen were noted, compared to tests performed with regular antigen on milk specimens to which the sodium bicarbonate solution had been added.

pH. The effect of pH was determined by ring testing milk at various pH levels from a high of 6.8 to 4.2. No effect was observed above pH 4.7, the iso-electric point for milk proteins. Coagulated milk samples, however, could not be read because of the lack of cream rising capacity.

Results of Ring Testing Milk From Strain 19 Vaccinated Cows

A total of 42 vaccinated cows were included in the study designed to determine the effect of vaccination upon ring test reaction. Time elapsing between vaccination and sampling ranged from 2 to 40 months for adult vaccinated cattle and 18 to 45 months for calfhood vaccinates, of which six were negative and two positive with a (+++) and (++++) reading. Both of the positive samples were from cows

which had been lactating for nine and 10 months, respectively. Results of ring tests performed on samples from 30 random selected cows are shown in Table 10. As evidenced by the table, very little agglutination inhibition was noted with samples from vaccinated cows when tested in the regular manner. The pre-warming technique when performed on these samples brought about several reductions in reactions.

TABLE 10 -- RING TEST REACTIONS ON MILK FROM 30 S-19 VACCINATED COWS

Cow	Vaccination Prior to Test (Months)	Entire Sample	Diluted 1:1 0.85% Saline
1	18	+++	++++
2	18	+++	++++
3	18	+	+
4	18	+	+
5	18	+++	++++
6	18	-	+
7	18	++	++++
8	18	-	-
9	18	++++	++++
10	18	++	+
11	18	+++	++++
12	18	+++	+++
13	18	++++	++++
14	18	-	-
15	18	-	+++
16	2	-	+
17	2	+	+++
18	2	+++	+++
19	2	+++	++++
20	27	++++	++++
21	40*	++	++
22	40	+++	++++
23	40*	++++	++++
24	40	-**	-**
25	40	+++	++++
26	40*	-	-
27	Calfhood	-	-
28	Calfhood*	+++	+++
29	Calfhood	-	-
30	Calfhood	-	-

Cows clean when adult vaccinated. Blood test positive cows also in herd at time of test.

*Lactation period of 9-11 months at time of sampling.

**N. C. = No cream.

DISCUSSION

Production of a specific colored antigen is by far the most critical step involved in the ring test procedure. Observations in this study indicate selection of a smooth *Brucella abortus* culture is of paramount importance and not too difficult to accomplish if proper medium and technique are employed.

Differentiation of the variants existing between the rough and smooth antigenic types brought about by the presence of sodium acetate in the growth medium was very difficult. Use of the colony typing techniques and the modified Albimi *Brucella* agar with subsequent colonial staining was helpful in distinguishing between R and S types. It was impossible to draw definite conclusions as to colony types resulting from the influence of acetate in growth medium through the use of these techniques.

Colonial morphololgy resembled the mucoid phase. It is reasonable to assume sodium acetate influences development of an antigenic type, which resembles neither the R nor S, since respective lots of antigen prepared in exactly the same mechanical manner differ greatly in ring test reactions.

Lack of reactivity exhibited by the antigen prepared from a rough culture may have been due either to lack of cell staining or loss of specificity. The latter reasoning is probably the most logical. However, since the antigens in this study were all stained in a standard manner, it would seem to indicate that the antigen specificity was decreased with the rough culture preparations.

Elimination of false positive ring test reactions by preliminary sample heating where the non-specific antigen was used seems to indicate these surplus positives were brought about by the Schern-Gorlisch reaction. Hanging drop preparations of non-specific antigen and previously determined false positive milks revealed cell agglutination with no apparent attachment to the fat globule. Similar preparation using a specific antigen and strongly positive ring test milks showed complete cell agglutination with some fat globule attachments, as evidenced by close proximity of antigen clumps and fat globules, coupled with a loss of Brownian movement of the antigen clumps. These observations would indicate that the Schern-Gorlisch reaction is dependent upon a simple filtration process, whereas the specific ring test reaction is a combination of filtration and actual antigen-fat globule attachment.

From results obtained, an apparently specific ring test reaction may be derived with a non-specific antigen when the preliminary sample heat treatment is employed. However, antigens vary greatly in speci-

ficity and this may not be true where a truly non-specific antigen is employed.

In the study of the effect of ageing of negative milk samples under refrigeration, a comparison was made of regular ring test procedure with the preliminary sample heat treatment using a specific antigen. With both test procedures a rather high initial false positive percentage was observed on reactions of fresh milk from individual cows. It should be noted, however, that in each method, the majority of the reactions are of a one plus (+) reading. Figure 3 shows 7.0 percent one plus (+) reactions out of a total of 10.3 percent surplus positive reactions on the fresh unwarmed milk. Results of the preliminary heated group, Figure 4, gave 7.1 percent one plus (+) reactions out of a total of 8.8 percent positive readings. Any slight color in the cream ring was read as one plus (+) reaction, which may have been somewhat severe; however, the figures agree rather closely with those reported of false positive reactions occurring on samples from individual cows negative to the blood test.

The sharp increase of false positive reactions brought about by ageing samples apparently is the result of increased clustering of fat globules. These clusters act as filters carrying the antigen along with them as they rise to the surface, which in effect, is a manifestation of the Schern-Gorlisch reaction.

Ability of the 45° C. sample warming, prior to testing, to dispel false positive reactions induced by ageing may be explained by dispersion of fat globule clusters. It has been reported that warming of milk to 50° C. will cause clusters to disperse into individual globules. This is brought about by causing a desorption of globulin.

Heat treatment of 45° C. will not correct all false positive reactions brought about by ageing of samples at refrigeration temperatures. This is indicated by Fig. 4 which shows a 21.3 percent increase in false positive reactions at the end of the four-day storage period. Perhaps this could be corrected by increasing length of the warming treatment or by greater agitation of the sample after heating, with subsequent cooling to facilitate creaming.

Specificity of the ring test upon positive samples was not altered appreciably. Five samples of positive herd milk decreased one plus (+) in reactivity; however, in each case these specimens represented samples taken from the cans at the receiving platform, which had been cooled and may have had several hours of ageing. This portion of the study was limited by the small number of known positive herds and individual cows which had been blood tested recently.

Reductions in intensity of ring test reactions brought about by the warming procedure were greater with individual positive cow samples than with positive herd samples. This can be attributed partially, at least to an intensification of the prozone phenomenon, as caused by heating antisera.

Freezing of milk samples prior to the conduct of the ring test was found to produce a large number of false positive reactions. It was observed that the false positive reactions increased both in intensity of reading and in total numbers as the freezing treatment became more severe. Close comparisons found between results of freezing samples and those of ageing seem to indicate that fat globule reactions were involved. This reasoning is further supported by a comparison of results obtained from heat treatment of samples prior to ring testing.

Apparently the effect of fat solidification by low temperatures is as responsible in causing false positive reactions as fat globules clumping and packing through ageing. Both factors are thought to be involved and according to the literature cited in the discussion on ageing, both should be eliminated by the preliminary warming technique.

From results obtained, it is indicated that the ring test can be modified to include detection of *Brucella* agglutinins in body fluids other than milk. Detection of antibodies in bull semen gave encouraging results with the limited number of samples available from blood test positive bulls. Since it was found that the ring test will readily detect agglutinins in positive blood samples, it follows that this should also be true for positive semen. Several samples of negative semen included in the results were from bulls which, although negative at the time of sampling, had given at one time or another blood test titers of complete at 1:50 and occasionally incomplete at 1:100.

It has been reported by numerous investigators, that brucellosis can be transmitted by artificial insemination with infected semen. It would seem that the modified ring test could be used as a quick screening test for detecting infection in unkown lots of semen and even in further checking of sires to be used for artificial insemination purposes.

No attempt has been made to correlate the modified ring test reaction of blood samples with the corresponding blood agglutination titer. It was noted, however, that with the usual milk ring test incubation period the modified ring test reaction was slightly less than the titer established with the blood test. This would be expected to vary slightly with different milk diluter sources. An arbitrary ring test reading for body fluids other than milk must await a large scale ring

testing of positive blood samples. These standards could then be applied to semen ring testing.

Ring testing of citrate-egg yolk diluted semen presents a confusing problem since it was found that false positive results are brought about by influence of the diluting fluid. Addition of calcium ions was found to overcome this false positive reaction, although whether this would cause a decrease in sensitivity of the modified ring test in detecting positive semen is not known.

Detection of blood serum agglutinins with goat's milk, which has little creaming ability, is possible with the ring test, although not too satisfactory. The detection of agglutinins in infected goat's milk would be rather uncertain with the ring test in its present stage of development.

It was found in this study, however, that a 1:1 dilution with negative cow's milk gave good results in detecting added agglutinins. From these observations, then, it should be possible to detect agglutinins in milk from infected goats if the cow's milk dilution technique is employed.

Formation of antigen clumps, appearing on the sides of the ring test tube where goat's milk had been employed for detecting agglutinins in blood, was observed after several hours incubation.

The whole milk dilution technique as described by Holm, gave the most satisfatory results of all diluters investigated. This technique overcame both problems of prozone phenomenon and faulty cream line, which are frequently encountered in testing individual cow samples.

Dilution at rates of 1:1 and 1:2 milk specimen to physiological saline gave results closely approximating the whole milk dilution technique except where poor creaming was encountered. This problem was improved by the saline dilution but not to the extent found with whole milk. Rapid formation of clearer, larger cream lines with a 1:1 saline dilution, compared to whole milk dilution, was found with normal samples.

The frequent demonstration of the prozone phenomenon, as demonstrated by a strong positive ring test with diluted milk with a corresponding negative reading on the undiluted portion, is in agreement with Holm, *et al.*, (1950), and Moore (1951). The latter reported this phenomenon was observed to occur in milk from six cows out of a total of 22 infected animals studied.

Intensification of the prozone phenomenon by the sample prewarming technique of the undiluted milk is confusing. It was thought at first that the temperature of milk at the time of antigen addition

was responsible. But, when samples were tested fresh at approximately the same temperature or warmer this was not observed. Apparently, agglutinin activity was increased by preliminary heat treatment of 45° C. for five minutes. This explanation seems logical since the pre-heated diluted samples occasionally gave a stronger ring test reading than corresponding non-heated undiluted portions.

From results obtained, it appears that a dilution of 1:1 physiological saline to milk dilution is the most favorable dilution to employ from a creaming standpoint. This dilution also appears adequate to overcome the prozone phenomenon in most cases. Use of tap water or distilled water in lieu of physiological saline gave comparable results. Use of zeolite softened water was found to induce a large number of false positive reactions. This situation apparently is due to a non-specific agglutination brought about by dissolved salts in the water.

Influence of citrate and oxalate ions upon ring test reaction was exceedingly interesting. At first it was thought that the effect might be due to exclusion of the calcium ion from the mixture, since addition of calcium chloride or calcium nitrate would overcome the influence. This idea was discarded when it was observed that tartrates did not bring about false positive reactions and, moreover, those reactions produced with citrates and oxalates could be overcome with other cations, including aluminum.

The theory of non-specific agglutination due to the influence of certain salts holds some promise as an explanation. It has been reported that non-specific agglutinations brought about by salts are influenced by cell sensitization through the adsorption of serum solids on the cell wall. Salts involved in various reports were cations whereas the salts responsible in this study were anionic in nature. Various unrelated substances have been reported to bring about non-specific agglutination of *Brucella* antigen and this may be the case with citrates and oxalates. Influence of semen, egg yolk, or egg white when added to mixtures containing citrates or oxalates also might be attributed to increased viscosity.

An excess of certain polyvalent anions plus the presence of certain serums which are found in colostrum, seem likely to be responsible for the false positive reactions on colostral milk, as reported by numerous investigators. This combination of salts plus serums also could be present in some normal appearing milks from individual cows. Sommers states there are cases where citric acid content of milk from an individual normal cow was found to be 0.4 percent in comparison to the normal of 0.2 percent. This might well be an explanation of the relative accuracy of the ring test on pooled milk, whereas, on the undiluted individual cow sample, specificity is not so great.

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SUMMARY AND CONCLUSIONS

1. Non-specific ring test reactions on milk brought about by ageing under refrigeration, freezing, or certain antigen preparations can be largely overcome by heat treatment of samples prior to testing.
2. Warming of samples prior to testing will not appreciably alter the ring test specificity for positive milks.
3. The ring test can be modified to detect agglutinins in blood and semen.
4. Whole milk dilution of samples is preferred for ring testing individual cow samples. However, physiological saline can be substituted except in some cases where poor creaming is encountered.
5. Laboratory diagnosed mastitis and sample pH has little or no effect upon the ring test specificity. Certain dissolved salts in milk will bring about non-specific reactions.
6. The majority of milk samples from adult vaccinated cows will give positive ring test reactions.

Report on Department of Dairy Husbandry
Research Project 139, Entitled
"Milk Production"