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Final Report:

BACTERIAL AND LEUCOCYTIC CONTENTS AND ENZYME LEVELS IN MILK AS METHODS FOR THE DETERMINATION OF MILK QUALITY AND FOR THE DETECTION OF MASTITIS

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Introduction

There are numerous reports on the subject of mastitis, and the relationship to somatic cell content, bacteria in milk, their quantitation and evaluation of milk quality and udders health.

1. Considerable disagreement prevails concerning the accuracy of the methods used for counting cells in the milk, the number of cells in normal milk, what consititutes an abnormal milk and what are the best methods for the determination of the parameters for the abnormal milk.
2. Tests for mastitis and milk quality are based on the physical examination of the milk and culture methods for pathogenic bacteria (1, 6, 9, 10).

   At present the tests consist of several approaches:

   a. The detection of substances in the milk which normally are not present or present in small quantities only and which appear as a result of bacterial activity or inflammation;

   b. Identification of changes in milk composition and physical characteristics resulting from the alterations in blood-milk barriers caused by the inflammation.

In the present research project these approaches were maintained as well. However, the following additional principles were stressed:

1. Utilization of newly developed technologies with greater sensitivity and specificity.
2. Speeding up the results obtained and cutting down the time to reach the needed information for the identification and quantitation of the inflammation and the causing pathogens.
3. Simplification of the test procedures.

The bacteriological tests, using present conventional methods for the identification, are time consuming and costly, but are important for the identification of the pathogens, for the proper treatment and control of the disease. The bacteriological tests, using present methods are time consuming

and consuming and costly, but are important for the identification of the pathogens for the proper treatment and control of the disease.

It was one of the objectives of this research project to evaluate the possibility of rapid, specific identification of the bacteria in milk, thus saving time and enabling an immediate start of treatment. One of the approaches used was by immunochemical methods, in which a specific surface antigen of the bacteria labeled with an enzyme marker, is added and complexed with the specific bacteria's antigen and enable its identification and quantitation.

The following procedures (based on the same principle) were evaluated.

a. ELISA in plates

b. ELISA on a filter or membrane - modified ELISA

c. Centrifugation

The various methods were designed to find a way in which the detection and identification of the bacteria will be rapid and specific and could be run in large volume. The procedure using centrifugal force to separate the complexed particle - conjugate, was applied also to leucocytes for their estimation to evaluate the leucocytes content in the milk and the degree of inflammation. For the determination of albumin in milk, conventional ELISA using plates was used only.

In another set of experiments, the approach of chemical - biochemical reactions for specific parameters was used. The analytes measured were for products formed by the pathogens, from leucocytes present as a result of inflammation or substances originated from the blood, following the changes or destruction of blood-milk barriers.

The various approaches and methodologies were tried and evaluated for their possible uses. The advantages, disadvantages, conclusions and recommendations are given.

Methods and materials:

The following experiments were conducted:

A. Immunochemical based tests.

1. conventional ELISA, using plates

2. modified ELISA using activated membranes - filters

The above were used for the detection of both bacteria and leucocytes, as well as other antigenic molecules such as albumin.

3. sedimentation of complexed antibody-antigen by centrifugal forces and measuring the activity of the marker enzyme.

B. Monitoring changes in milk composition following products originating from bacterial activity, enzymes or other substances produced during mastitis.

The effects of the milk itself (naturally occurring substances which affect the various test reactions (turbidity, lipids, enzymes etc.) were determined to allow optimization of the test procedures and modifications needed to overcome these problems. A comparison of antigen detection in saline vs. milk was done.

C. Adaptation of existing tests commonly used in other fields, for the evaluation of milk quality and the presence of mastitis.

A. Conventional ELISA, using plates - was done according to Clark and Adams (3). Antibody coating were done at concentrations of 5.0 µg protein/well.

Testings were done with the following groups:

a) control milk samples, having no mastitis, or indentification of any pathogenic bacteria.

b) milk samples taken from commercial farms, showing positive mastitis and presence of pathogenic bacteria (as identified in the department of udder diseases, Kimron Veterinary Institue)

c) bacteria samples, isolated from milk samples, identified, grown, harvested and suspended in phosphate buffered saline.

Samples containing different bacterial counts (obtained by dilution with saline) were studied. Different concentrations of conjugates were used to establish the optimal conjugate concentrations.

Para-nitrophenylphosphate (Sigma Chemicals, USA) was used as the substrate and the color formed was read with the Dynatech ELISA reader model MR 5000 at 405 mμ.

In experiments, using the modified ELISA on membranes, the round discs of membranes (radius 6 and 12 mm) were incubated with the substrate in the plate. At the end of the reaction, the membrane was removed, the color in the solution was read with the ELISA reader and the color intensity on the membrane was estimated on a 1-4 scale.

Antibodies

Both polyclonal and monoclonal antibodies were used:

Polyclonal antibodies against E. coli - K 99 were obtained from colostrum of cows immunized with the above strain. The immunoglobulins were partially purified by ammonium sulfate precipitation followed by dialysis and lyophilization.

The preparation and extraction were done either in the department of immunology at Kimron Veterinary Institute, or in Kibbutz Maabarot producing the "Immunocol" (fractionated immunoglobulins against E. coli - K 99 according to procedures and technology developed in the department of immunology).

This material was used for the various immunochemical studies conducted.

Monoclonal antibodies against leucocytes were produced and donated by A. J. Guidry, from the Milk Secretion and Mastitic Laboratories in USDA, Beltsville, MD, USA.

The following monoclonal antibodies were used:

Type	clone I.D.*	Isotype
A	49G4A2D2	G_1, K
B	50EGA4C10	G_1, K
C	23H9C306	M. Lamda
D	36H10E10F6	G_2, K

* according to the 1987 library of monoclonals to bovine PMN

Conjugation of antibody

Antibodies were conjugated with alkaline phosphatase according to Engvalle (4). The purified enzyme was purchased from Sigma Chemicals (St. Louis, Mo. USA).

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A1. Detection of bacteria and leucocytes by specific surface antigens using immunochemical methods

I. Detection of bacteria using a modified "dip stick" ELISA (enzyme linked immunosorbent assay). The "sandwich" ELISA for antigen was used (4, 8). The procedure used was as follows:

Antibody against a bacteria (E. coli - K99) was bound to an activated membrane (activation of membrane and types will be described later). Binding was done in 2 ways: (1) incubation of the membrane in a solution containing the antibody (2 mg protein/ml), overnight at 0-4°C; (2) application of antibody solution to the activated filter and letting it dry at room temperature.

Following the binding, the membranes were washed and the open spaces were blocked with 2-3% of albumin or casein solutions. Following the incubation with the blocking solution, the filters were washed and stored until testing at 0-4°C.

Testing procedure - the filters were dipped in a solution (PBS or milk) containing the bacteria followed by washing, adding and incubating with the conjugated antibody, followed by another washing and addition of the substrate. At different times during the incubation, color intensity was estimated both visually and colorimetrically (using a spectrophotometer or ELISA reader).

Alkaline phosphatase was used as a marker for the conjugated antibody and para-nitrophenyl phosphate was used as the substrate reading at 410 mμ.

For the evaluation of the results on the filter = dip stick, a conventional ELISA was run in parallel.

Detection of E. coli in saline and milk with ELISA using Biotin-Strepavidin-peroxidase conjugate.

Using the Biotin-Strepavidin peroxidase conjugate for the detection of E. coli suspended in saline and milk (5), showed the plates method useful at concentrations of bacteria above 10^4/ml. Using membranes instead of plates resulted in significantly higher readings in milk, making it a potentially better and more sensitive procedure.

The test procedure however is more elaborate and the reagents are more expensive, factors which have to be taken into consideration while choosing the procedure of choice for the detection of bacteria in milk.

The following variables were tried:

1. concentrations of bacteria in the solution ranging from 10^1 - 10^8/ml
2. bacteria in milk and saline
3. various concentrations of conjugated antibodies
4. various kinds of bacteria to evaluate the specificity and cross reactivity
5. Effect of heating the tested samples (70°C, 15 min) on the antigenicity of the bacteria and leucocytes (this experiment was conducted to assure that any residual enzymatic activity present in raw milk was abolished to reduce possible background activity present in raw milk).

A2. Filters - activation and types

To assure the selection of the optimal filter, giving the best binding with the lowest background activity, a wide range of filters and membranes were tried (7, 11).

Two main subgroups were evaluated:

1. cellulose and nylon membranes activated by us (with the aid of Prof. L. Goldstein, Department of Biochemistry, Tel-Aviv University, Israel - a specialist in membrane chemistry).
2. those obtained from the industry (Gelman Ltd. - USA).

The following table depicts the various filters.

Binding of proteins to membranes was done in both methods: overnight incubation at 0-4°C and immobilization by application and drying.

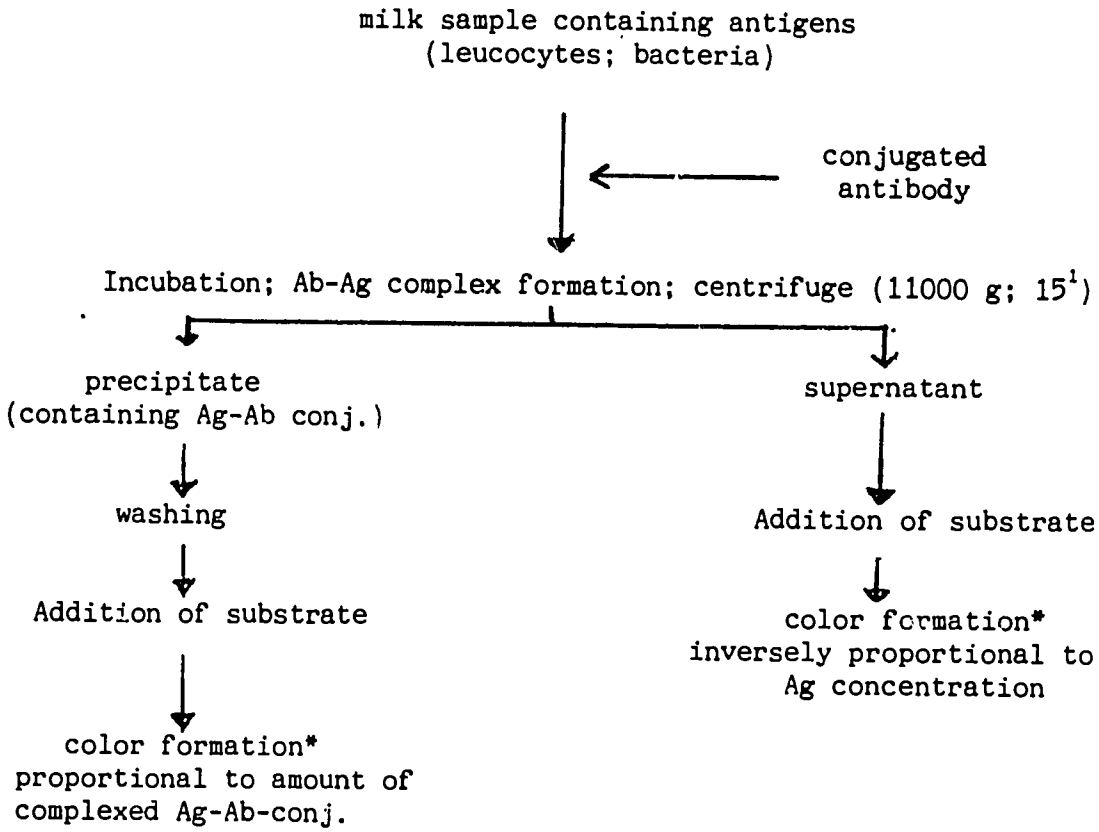
Various filters and membranes screened for the "ELISA - Dip Stick"

1. Nitrocellulose - protein activation via NH_2 terminal
2. Nitrocellulose - protein activation via COOH terminal
3. Nylon - protein activation via NH_2 terminal
4. Nylon - protein activation via COOH terminal

Whenever activation was done via the NH_2 terminal, the protein was suspended in a acetate-buffer (acidic pH). Whenever the activation was done via COOH terminal of the protein, it was suspended in tris-buffer (basic pH).

5. Gelman - NT, pore size 0.5 µm (with a nitrocellulose binding material)
6. Gelman - RP, pore size 0.45 µm
7. Gelman - ultrabind affinity membrane, pore size 0.45 µm
8. Gelman - Biotrace RP (charged nylon), pore size 0.45 µm.

B. One of the proposed methods for the detection of antigens is the addition of the antibody conjugate directly to the milk and centrifuge it following the formation of the antibody - antigen complex and read the ALP activity in both the supernatant and sediment.



Reaction was stopped by the addition of NaOH.

The advantage of this method is its simplicity, speed and low cost since the method does not require the steps commonly used in the conventional ELISA procedures (protein binding to solid phase, washing of excess conjugates, blocking etc.).

Bacterial antigens used were E. coli - K 99 with the antibody obtained from colostrum as previously described.

Since raw milk contains high concentrations of the enzyme ALP, the milk samples were heat treated (60°C, 25') prior to the addition of the antibody conjugate.

Since the balance of antigen to conjugated antibodies is critical, low and high concentrations of conjugated antibodies were tried.

C. Detection of bacterial activity using tetrazolium - TTC

The method was designed to develop and evaluate a screening test for the presence of bacteria in milk. This method, which originally was described for the detection of bacteria in urine samples, was adapted by us for milk. The principle of the test is that triphenol tetrazolium chloride (TTC) is reduced, forming a red-purple color (2).

Detection of Bacterial Activity by Nitrites Determination with Urinalysis Strips

The test for milk is an adaptation of an existing test in urinalysis for the detection of bacteria. The test (Griess test) is used with the urinalysis strip (Boehringer-Mannheim - Combur test-U). Bacterial present in milk will reduce nitrates (NO_3^-) to nitrites (NO_2^-). Nitrites is commonly present in the milk in small quantities, but additon of NO_3 is preferred for clearer results.

Detection of leucocytes in milk using Urinalysis Strips

A common test for urinary tract inflammation is by the detection of leucocytes in the urine. The test is based on the determination of esterases present in leucocytes.

The esterase breaks down an indoxyl ester, releasing free indoxyl which then reacts with tetrazolium to yield a blue-violet color.

Appearance of the color is an indication of presence of enzymatic activity and leucocytes.

Results and Discussion

Using the immunochemical based reactions for the identification and quantitation of large particles such as bacteria or leucocytes in milk, showed that in principle the methods can be adapted for use with milk for the evaluation of milk quality and presence of mastitis.

Detection of E. coli - K99 suspended in saline and milk was possible with the conventional ELISA. Higher optical densities, indicating a better reaction were seen whenever the E. coli was suspended in saline in comparison to E. coli suspended in milk. Dilution of milk with saline x(5-10) improved the reaction, yielding a stronger signal (Table 1).

Positive readings were obtained with high bacterial concentrations (10^5-10^7/ml), either in saline or diluted milk samples (Table 1). Under the given conditions, this procedure is capable to detect as littl. as 10,000 bacteria. At lower bacterial count, even though some color was developed, it was relatively small.

It was possible to increase the signal by longer incubation time, but this is not optimal and other ways to increase the sensitivity should be established.

Detection of E. coli with the modified ELISA, using activated membrane or filters as the solid binding phase showed to be more sensitive and potentially more practical and applicable (Table 2).

Significant readings were obtained with bacterial count of 10^3 (or 1000 bacteria), whenever the filters to which the bacteria and conjugates were complexed, were incubated with the substrate in a test tube or ELISA plate and removed prior to the reading of the color.

Reading the color developed on the membrane following the incubation with the substrate showed it to be sensitive, as well as positive (Table 2) detectable results at bacterial count of 10^4/ml. (Table 2).

These results suggest that using a modified ELISA with activated membrane may have an advantage because of greater sensitivity and the potential for the development of "dip stick" system for the detection of specific bacteria with an estimation of their concentration in the tested fluid.

As seen in Table 3, little to no cross reactivity between E. coli - K99 and non K99 E. coli, (Salmonella and Staphyloccocus) were seen. These data, strongly support the possibility that the proposed method can be used for the specific identification and quantitative estimation of bacteria in milk.

Results in Table 4 suggest that blocking the open unoccupied spaces in the ELISA reaction well will improve the reaction, increasing the sensitivity (more so in milk than in saline samples). This was more pronunced with lower bacterial count.

A similar pattern was seen whenever activated membranes in the modified ELISA method were used, producing significant higher readings whenever the bacteria was suspended in milk (Table 5).

Results on the detection of E. coli using Biotin - streptavidin conjugated to peroxidase for the ELISA, either in plates or with membranes are given in Table 6.

Using this procedure in ELISA plates showed it to work whenever the bacteria was suspended in saline at concentrations above 10^4/ml (which was somewhat more sensitive in comparison to the ALP conjugate). Whenever the bacteria was suspended in milk, positive readings were obtained only at a very high bacterial count (10^8/ml).

The results obtained using the modified ELISA procedure with activated membranes showed once again that this procedure yields better results with greater sensitivity, making it a potential preferred method. Furthermore, results obtained with milk samples showed to have little or no inhibition by milk as seen with the conventional ELISA plates. Using activated

membranes resulted with a good signal even with a bacterial count of 10^4/ml.

Coating beads with E. coli antibodies and following the agglutination which takes place in the presence of various concentrations of E. coli showed the system to work very well, with sensitivity at levels of as low as 100 bacteria/ml (Table 7).

Reading was possible only whenever the bacteria was suspended in saline. It was not possible to follow turbidity formation with milk samples because of the very high initial turbidity of the milk (Table 7).

Two different types of beads were used, yielding similar results. Best results were obtained whenever coating was done with high antibody (undiluted) concentration.

In future experiments trials to overcome the interference by the milk turbidity should be done. One possible way is by prior sedimentation (by centrifugation) of the bacteria and suspension in a non-turbid solution.

The overall impression is that whole bacteria can be detected by ELISA provided the antibodies used are against the bacterial surface antigens. Better results were obtained whenever the modified ELISA was used. This was probably due to the possibilities that with activated membranes (1) washing of the turbidity contributing substances was better (2) washing of other inhibitors to the reaction (3) binding of the bacteria to the antibody bound to the membrane was better and stronger due to the three dimensional structure of the porous membrane. This structure of the membrane, prevented the vigorous washing and removal of the bacteria which is a very large particle. The three dimensional structure probably allows a binding of the bacteria in more than one site, thus giving it a stronger binding to the membrane, preventing its detachment during the washing process.

Detection of leucocytes for the evaluation of mastitis and milk quality are shown in Tables 8-12. As seen, significant higher readings were observed, whenever ELISA was performed with 4 different monoclonal

antibodies against leucocytes. While the readings were less than 0.05 OD in 3 out of the 4 antibodies, it ranged from 0.171 to 0.537 OD in mastitic milk, containing high levels of leucocytes, an increase of up to 1000 percent (Table 8).

In order to increase the sensitivity and lower the background activity of ALP (an enzyme naturally found in fresh milk), heat treatment prior to the ELISA was evaluated. The results obtained showed that following heat treatment at 60°C for 20 minutes, the antigenicity of the leucocytes changed resulting in a failure of antigen-antibody complexing and consequently loss of activity (Table 9).

Detection of leucocytes in mastitic milk induced by E. coli, S. agalactea and Staphylococcus using leucocytes monoclonal antibodies is shown in Table 10. Modified ELISA procedure using activated membranes was performed.

As seen leucocytes were detected in mastitic milk regardless of the causing pathogens. It seems that different levels of leucocytes were present in the different milk samples, as seen from the different readings of the various samples (Table 10). Detection of leucocytes by the modified ELISA procedure, using activated membranes, following dilution of the mastitic milk, showed lower readings with dilution (Table 11). It may be assumed that the lower readings were in response to the smaller number of leucocytes present following the dilution. A positive relationship between color intensity and number of leucocytes was seen. A similar pattern with all 3 tested antibodies was seen (Table 11).

The effect of milk sample freshness on the detection of ELISA is shown in Table 12. As seen following storage of milk samples significant loss of sensitivity occured. This could be related to the loss of the antigenicity due to denaturation or any other possible structural changes of the antigen. Furthermore it could be related to the destruction of the cells and unfolding of the membrane, making the surface antigen less available

for interaction with the antibodies.

The "centrifugation" approach is based on the hypothesis that whenever a conjugated antibody binds, antigen-antibody complex will sediment by a centrifugal force. If a known amount of a conjugated antibody is added to a suspension containing large particles such as bacteria or leucocytes, the amount of conjugated antibodies complexing to the antigen will be proportional to the number of antigenic particles and consequently less conjugated antibodies will remain in the supernatant and more will precipitate. The amounts left in the supernatant and precipitating can be measured by monitoring the activity of the conjugated enzyme (alkaline phosphatase, peroxidase and others).

The results of the experiments evaluating this approach are shown in Tables 13-17. Table 13 describes the activities (expressed in OD units) of the conjugated enzyme in the supernatant and precipitate following the incubation with normal and mastitic milk. Two types of antibodies were used. As seen, only one antibody (B) yielded acceptable results, giving higher readings in the precipitate and lower readings in the supernatant of mastitic milk. Results shown in Table 14, evaluating the optimal concentration of antibody conjugate in the reaction with leucocytes in milk showed that a 1000-2000 dilution of the stock is optimal for best color development. Whenever presence of E. coli was studied in milk, using the centrifugation procedure, best results were obtained with a dilution of $2x10^4$ of the conjugated antibodies. Whenever color development was estimated in the supernatant, relatively lower concentration of conjugated antibody is preferred to avoid the development of very strong color (Table 15).

Measurement of color development in the precipitate following incubation and centrifugation of E. coli in saline showed it to be more sensitive than measurements of the supernatant, obtaining readings even with bacterial count of 10^3/ml. The conditions of such an experiment

allowed a higher concentration of conjugated antibodies (Table 16).

The effect of milk dilution with saline on color development of the precipitate, following incubation and centrifugation of E. coli suspended in milk, are shown in Table 17. As seen dilution of milk improved sensitivity of the test, once again suggesting the possible presence of an inhibitor in the milk affecting the reaction (Table 17). In this case it may be worthwhile to dilute the milk sample whenever a greater sensitivity is required.

One of the approaches to detect poor quality, and mastitic milk or milk containing high levels of bacteria or stored under improper conditions was by following a product of bacterial activity in the milk. One such activity is the breakdown of urea (normally present in the milk at concentrations similar to those of the blood) to ammonia (NH_3). Normally, ammonia is present in the blood and milk in small quantities. In the body (liver) it is being condensed together with CO_2 to urea, while the bacteria is doing the opposite reaction.

$$2NH_3 + CO_2 \xrightleftharpoons[\text{Bacteria}]{\text{Liver}} H_2N - \overset{\overset{\displaystyle O}{\|}}{C} - NH_2 + H_2O$$

It was therefore postulated that monitoring levels of ammonia in the milk could serve as test for milk quality and presence of bacterial activity. Tables 18-20 describe the results evaluating this method.

Ammonia concentrations in milk from normal and mastitic udder are shown in Table 18. As seen, depending on the degree of inflammation (as evaluated by California Mastitis Test - CMT), ammonia concentration is greater. While milk from healthy udder and pasteurized milk contain less than 400 µmol/l, in mastitic milk it ranged from 600 to about 4000 µmol/l, a 10 fold increase, making it a potential good screening test for mastitis and poor quality milk (Table 18).

Ammonia production in milk in relation to bacterial count is shown in Table 19. As seen, there was a linear relationship between the bacterial count in milk and NH_3 concentration following the addition of E. coli to pasteurized milk and incubation for 15 minutes (Table 19).

Keeping fresh milk at room temperature led to increased production of NH_3, increasing from 340 µmol/l at 0-time to over 8000 after 24 hours, clearly showing the usefulness of this method for the evaluation of milk storage conditions and quality (Table 20).

Monitoring NH_3 concentrations in milk either fresh or processed seems to be a very promising tool to establish mastitis, milk quality, storage conditions and possibly presence of antibacterial substances in the milk. The possibility to couple it to a simple test can make it a qualitative as well as quantitative test, which can be performed either on the field or in the farm, in the laboratory, factory or as needed in various inspection sites. It is rapid and inexpensive and should be introduced for routine uses.

Another approach used in the present research project was to evaluate the potential of special enzymes as markers for bacterial activity or inflammatory processes in the udder and consequently in the milk. As seen, most enzymes studied do change as a result of inflammation in the udder. Changes in enzymatic activities are a result of udder cellular destruction, lymphocytes, blood (following blood milk barrier destruction) and bacteria.

Accordingly, it is pssible to divide the various enzymes in the milk into several groups with indication to bacterial presence, inflamation and presence of leucocytes. For a more sensitive and better diagnosis, it is preferred to use parameters which show a dramatic change, e.g. from no to little levels up to a pronounced concentration as compared to parameters which normally exist at relatively high concentrations and further increase as a result of the inflammatory process.

Into the first group, enzymes like CK, ALT, and amylase are good

representatives, while in the second group representing enzymes are acid and alkaline phosphatase, AST and LDH. Of interest is the fact that while acid phosphatase increases during mastitis, the enzyme alkaline phosphatase decreases. Presently it is not clear what is the origin of the enzyme CPK in the mastitic milk It is possible that some of the activity originates from the blood. However, it can not explain the relatively high activities seen. Since the test for the determination of CPK based on the generation of ATP, it is possible that ATP or other systems capable to generate ATP are present in the mastitic milk, which give the CPK signal.

For further evaluation, a commercial kit (APIZYM) used for the identification of bacteria was tried for milk. (Table 22).

APIZYM is a commercial kit used for the screening of unpurified complex samples, providing a spectrum of enzymatic determinations in the specimen tested. APIZYM is a semi-quantitative micromethod system, designed for the research of enzymatic activities in the tested sample. The technique is applicable to all specimens (tissue, cells, biological fluids, microorganisms, washings, soil, oil etc.). It allows the systematic and rapid study of 19 different enzymatic reactions.

Milk samples were taken from normal and mastitic udders, the CMT was conducted and the presence and identification of pathogenic bacteria was determined by conventional bacteriological methods. Milk samples were then tested with the APIZYM kit, using the following groups: a) normal - pathogenic bacteria free, b) mastitic milk containing Strep. uberis, c) mastitic milk containing E. coli, d) mastitic milk containing micrococci, e) mastitic milk containing staphylococcus, f) pasteurized milk.

Comparing the results obtained with mastitic milk containing the various types of bacteria to those of control milk, containing no detectable pathogenic bacteria, suggest on possible use of some enzymes as indicators for specific bacteria presence in milk (Table 22). The enzyme leucine arylamidase showed to be significantly more active in mastitic milk

containing staphylococcus. Also the activity of the enzymes alpha-glucosidase and N-acetyl-B-glucosaminidase were much higher in mastitic milk containing staphylococcus. Furthermore the enzyme N-acetyl-B-glucosaminidase showed to have higher activity in mastitic milk samples containing streptococcus uberis.

Using the APIZYM kit for the evaluation of milk and presence of pathogenic bacteria does not seem to have the answer needed for milk screening and identification of specific bacteria. It is too general, expensive and time consuming.

There are several existing tests presently used routinely for the detection of bacteria and leucocytes in urine. These tests are widely used in medicine, are commercially available and inexpensive, are simple to use, and give a qualitative - semi-quantitiative results within 1-2 minutes. We have tried to adapt them for use in milk evaluation and the results obtained are given in Tables 23, 24.

One such test is the detection of nitrites. This substance is a product of nitrates reduced by the bacteria. Nitrates, which are commonly found in men and animal urines serve as the substrate for the production of nitrites by the bacteria. Presence of nitrites are therefore an indication to bacterial activity. In ruminants, the amount of nitrates (NO_3) in the milk is low and NO_3 was added to assure sufficient substrate to produce nitrites (NO_2). The results obtained are given in Table 23. As seen milk from healthy udder showed no detectable levels of nitrites, while mastitic milk containing bacteria showed to have significant levels of nitrites, clearly suggesting that the test could be used with milk for the detection of bacteria and mastitis.

In order to assure that indeed the bacteria is responsible for the reaction producing nitrates, pasteurized milk into which bacteria (E. coli) and nitrates were added and used to mimic the events which take place naturally in the udder. As seen in Table 23, positive nitrite test in these

milk samples were detected, thus supporting the hypothesis of the nitrites origin and pathway of production. Freezing and thawing milk samples did not affect the test and thus it can be used on frozen samples as well.

The nitrite test can serve as an efficient fast test for the detection of bacterial activity in the milk. Since there is no need for any special development of the test and the strips are commercially available, one can easily adapt it for the purpose of milk screening as an independent or supporting test.

In another test, the diagnostic value of the enzyme esterase was evaluated. Esterase originating from leucocytes present in the milk as a result of inflammation (mastitis). As seen in Table 24, milk from healthy udder contained no esterase activity, while mastitic milk, containing both leucocytes and bacteria contained significant high esterase activity, which was lost following heat treatment (70°C; 10 minutes). Most of the esterase activity was associated with the particulate fraction, which was sedimented following a centrifugation.

The esterase activity test, commonly used in urinalysis for the detection of inflammation seems to be a useful marker for milk evaluation for the presence of leucocytes (and probably bacteria as well).

Both nitrites and esterase tests which are used as part of a bigger panel of screening tests in urinalysis, could be adapted for milk evaluation. Since the tests are commercially available, are simple inexpensive and fast, requiring the dipping of the test strip in the specimen and reading against a color chart, it is recommended to adapt their use in the routine screening of milk.

The reduction of triphenyl-tetrazolium chloride (TTC) is used in urinalysis for the detection of bacterial activity. The tetrazolium is reduced resulting in a change of color. The intensity of the color formed with time is proportional to the bacterial count and viability.

This test was performed with milk to evaluate its usefulness in

screening milk containing bacteria. As seen in Table 25, the use of this test for milk evaluation is possible, yielding a strong color signal with mastitic milk or milk containing bacteria (E. coli; S. aureus). Heat treated (pasteurization) or freezing, abolished the ability to reduce TTC. The test is simple, relatively fast and should be recommended for a routine screening test of milk contamination.

Loss of activity following freezing or storage may be related to the capacity to generate protons (H^+) for the reduction of tetrazolium, which causes the change in color.

Serum albumin is a protein which is found in large concentrations in the blood serum and only very small concentrations in the milk. As a result of inflammatory process, blood-milk barrier is changing and albumin from the blood moves into the milk leading to increased concentrations of milk albumin. Determination of albumin in milk therefore can serve as possible parameter for the presence of mastitis and degree of blood-milk barrier damage. As seen in Table 26, detection of albumin, using the conventional ELISA, gave positive clear signals from concentrations of 0.3 to 40 mg/ml of saline. It seems that the optimal albumin concentrations suspended in saline for the ELISA is about 1 mg/ml. Having higher concentrations of albumin led to inhibition in the reaction. Whenever the albumin was determined in milk having similar concentrations, lower optical densities were obtained. Unlike the results seen with saline, higher readings were obtained with higher albumin concentrations, with the highest reading at 40 mg/ml.

Blocking the spaces in the reaction well with casein caused some inhibition with samples suspended in saline, an inhibition which decreased with increasing concentrations of albumin. Whenever milk was tested the inhibition by casein was even greater, completely inhibiting the reaction. The reason for this inhibition could be the possibility that the bound albumin molecules are replaced by the casein molecules which are present in

excess more so in milk than in saline.

Albumin

Because of the destruction of blood milk barrier duirng mastitis, serum albumin which in normal healthy udder is present only in very small concentrations, significantly increases during mastitis. It was proposed in this research project to evaluate the usefulness of measuring albumin concentration as a parameter for the determination of the presence and degree of the udder inflammation.

Bovine albumin concentrations in milk were determined using the conventional ELISA. Bovine albumin antigen was purchased (Bio-Yeda, Rehovot, Israel) and a conjugate with alkaline phosphatase was prepared. Albumin was added to milk, to give different concentrations and the determinations were conducted under different dilutions and incubation times, to determine the optimal conditions. Both fresh and pasteurized milk (to eliminate background activity of alkaline phosphatase in raw milk as well as to evaluate the effect of heat treatment on albumin and its possible denaturation and change in antigenicity) were tested.

The need for blocking the open spaces on the solid phase in the plate following the attachment of the antibody to the reaction well was evaluated. Since albumin antibody was attached to the well wall, blocking was done with casein, prior to the addition of the milk samples.

As seen in Table 26 measurement of low concentrations of albumin in milk is not possible, probably because of some inhibition caused by a substance present in milk. It may be related to the high concentration of casein in milk, since some inhibition by casein blocking was seen. Some readings were obtained only at high albumin concentrations, levels which are too high for the routine use of albumin as a marker for mastitis.

In conclusion, several approaches for the detection of mastitis, evaluation of the degree of the inflammatory process detection and determination of the causing pathogen.

The guidelines in the research were to evaluate and establish procedures characterized by simplicity, speed and low cost, tests which can be performed both in the more sophisticated laboratory for quantitative results as well as under field conditions for a semi quantitiative - qualitative results.

The investigators tried to adapt existing tests, currently being used in other areas such as urinalysis, blood analysis, etc. The results obtained clearly showed that this can be accomplished, introducing new methods for the screening of milk samples, thus overcoming some of the major problems in milk and mastitis analysis, shortening the required test time, introducing more specific tests and thus cutting down the losses and diagnostic costs.

The present study is a basis for further research needed to cover the various hosts of pathogens and cases in mastitis diagnosis. Additional research is required for the analysis of other pathogens, producing specific surface antibodies and introducing it to the available panel of tests to allow the establishment of a multitest for the simultaneous detection of several bacteria and leucocytes, which can be constructed according to the conditions and needs of various regions in the world.

Being able to come with an answer to the questions of: (1) presence of mastitis, even at an early-preclinical stage, and (2) type of the causing pathogens, has a tremendous contribution to the field of mastitis diagnosis and early treatment. The results obtained in this research project seem to promote this goal.

Table 1 **Effect of Milk Dilution with Saline on the Detection of E. coli, using ELISA**

Media	E. coli Concentration/ml					
	10^7	10^6	10^5	10^4	10^3	10^2
Saline	1.30	0.50	0.38	0.05	0.05	0.07
Diluted milk (1:10)	1.90	1.40	0.46	0.06	0.02	0.02
Diluted milk (1:5)	1.80	1.20	0.38	0.04	0.00	0.00
Concentrated milk	0.76	0.10	0.08	0.00	0.00	0.00

Mean OD , n=5

Table 2 **Detection of E. coli - K 99 in Saline and Milk, Using a Modified ELISA on Filter Membranes**

	ELISA Reader					Visually			
	Bacterial concentrations / ml								
	10^7	10^6	10^5	10^4	10^3	10^7	10^6	10^5	10^4
Saline	2.9	2.7	0.9	0.8	0.6	+++	+	+	+
Milk	3.4	1.8	0.8	0.5	0.2	+++	++	++	+

n = 10; mean O.D. at 405 nm

Table 3 **Evaluation of Cross Reactivity Between E. coli - K-99 and other bacteria using the modified (membrane) ELISA.**

Bacteria	Concentration/ml						
	10^7	10^6	10^5	10^4	10^3	10^2	10^1
E. coli K-99	2.9	2.7	0.9	0.8	0.6	0.5	0.3
Salmonella	0.3	0.2	-	0.2	0.02	0.00	0.00
E. coli (non K-99)	0.40	0.28	0.33	0.26	0.00	0.00	0.00
Staphylococcus	0.00	0.00	-	-	-	-	-

Bacteria were suspended in saline. The above is an average of 10 tests. Activity is expressed as O.D. read with ELISA reader at 405 nm, following the incubation of the filter membrane in the ELISA plate.

Table 4 **Effect of Casein (3%) Blocking on E. coli Detection Using ELISA Plates**

Media	Casein treatment	Bacterial Count / ml				
		10^7	10^6	10^5	10^4	10^3
Saline	-	0.662	0.340	0.240	0.280	-
	+	0.681	0.628	0.175	0.168	-
Milk	-	-	0.339	0.00	0.00	0.00
	+	-	0.311	0.101	0.125	0.05

Mean activity expressed in O.D. at 405 nm ; n=4

Table 5 **Effect of Casein* (3%) Blocking on E. coli - K-99 Detection Using ELISA (modified) on Membrane**

Media	Casein treatment	Bacterial Concentration / ml				
		10^7	10^6	10^5	10^4	10^3
Saline	-	3.00	1.36	0.49	0.30	0.25
	+	3.00	2.55	0.215	0.20	0.19
Milk	-	2.90	0.73	0.49	0.20	0.07
	+	4.50	2.58	1.55	0.52	0.39

Mean activity expressed in O.D. at 405 nm following the color development in ELISA plate ; n=4

* similar results were obtained whenever blockage was done with 2% BSA.

Table 6 **Detection of E. coli using Biotin-Strepavidin-peroxidase conjugate with ELISA in plates and membranes.**

Suspension	Bacterial concentration /ml				
	10^8	10^7	10^6	10^5	10^4
saline - plate	0.625	0.625	0.711	0.231	0.062
saline - membrane	0.850	0.765	0.160	0.300	0.035
milk - plate	0.585	0.000	0.000	0.000	0.000
milk - membrane	2.370	0.450	0.435	0.325	0.155

Biotin-Strepavidin Peroxidase conjugate dilution 1:500 in plate and 1:1000 with filter discs. Blocking was done with casein.

Values are mean of 3 optical density at 450 nm.

Table 7 **Agglutination of Antibody Coated Beads by Varying Concentrations of E. coli Antigens.**

Beads	Concentration/ml					
	10^7	10^6	10^5	10^4	10^3	10^2
Type A (blue)	0.667	0.368	0.243	0.294	0.307	0.283
Type B (yellow)	0.490	0.230	0.200	0.187	0.180	0.199

Agglutination was measured by turbidity at 550 nm using ELISA reader. The results are an average of 6 experiments. E. coli were suspended in saline.

Table 8 **Detection of Leucocytes in Control and Mastitic Milk, With ELISA, Using Monoclonal Antibodies.**

Monoclonal antibody-type	Milk	
	Control	Mastitic
A	0.040	0.298
B	0.029	0.171
C	0.111	0.250
D	0.051	0.537

Milk was diluted 1:10 with saline. Results are average of 6 tests.

Table 9 **Effect of heat treatment (60°C, 20 min) on milk samples using ELISA (modified) for determining leucocytes in milk samples using 3 types of antileucocytes monoclonal antibodies.**

milk sample	monoclonal antibody		
	A	B	C
Control	0.00	0.00	0.00
mastitic - fresh	0.816	0.279	0.653
mastitic - heated	0.023	0.00	0.057

milk samples were diluted 1:10 with saline.

Table 10 **ELISA (modified) determining leucocytes in control and mastitic milk caused by various pathogens, using 3 types of antileucocytic monoclonal antibodies.**

milk sample	monoclonal antibody		
	A	B	C
Control	0.00	0.00	0.00
Mastitic - induced E. coli exp. 1	0.816	0.279	0.653
Mastitic - induced E. coli exp. 2	1.032	2.225	1.336
Mastitic - induced S. agalactea	-	0.528	0.866
Mastitic - caused by Staphylococcus	0.184	0.250	0.338

milk samples diluted 1:10 with saline.

Table 11 **Effect of milk sample dilution on ELISA (modified) results determining leucocytes in milk using 3 types of monoclonal antibodies.**

milk sample - dilution factor		monoclonal antibody		
		A	B	C
Control	10^1	0.044	0.027	0.038
Mastitis - E. coli	10^1	1.032	2.225	1.336
Mastitis - E. coli	10^2	0.282	0.242	0.227
Mastitis - E. coli	10^3	0.091	0.063	0.070
Mastitis - E. coli	10^4	0.082	0.062	0.056
Mastitis - E. coli	10^5	0.019	0.002	0.010
Mastitis - E. coli	10^6	0.000	0.000	0.005

dilution with saline

Table 12 **Effect of milk sample freshness on ELISA (modified), determining leucocytes in control and mastitic milk samples, using 3 types of antileucocytes monoclonal antibodies.**

milk sample	monoclonal antibody		
	A	B	C
Control	0.00	0.00	0.00
fresh	0.816	0.279	0.653
48 stores (4°C)	0.131	0.083	0.170

milk samples diluted 1:10 with saline.

Table 13 **Color Development in Both the Precipitate and Supernatant fractions, Using the Centrifugation Procedure for the Determination of Lymphocytes in Mastitic Milk.**

Conjugated Antibody	Optical Density			
	Supernatant[a]		Precipitate[b]	
	Normal	Mastitic	Normal	Mastitic
B	0.106	0.554	0.00	0.18
C	0.066	0.100	0.00	0.02

The values expressed in O.D. at 410 nm are:
a) the difference obtained by subtracting the test reading from the blank
b) the difference obtained by subtracting the blank from the test.

Milk samples were diluted 1:100 with saline. Milk samples were not heated.

Table 14 **Effect of Antigen (E. coli) and Conjugated Antibodies Concentrations on Color Development of the Precipitate - In Milk**

dilution of Ab-conj	E. coli concentration /ml				
	Blank	10^7	10^6	10^5	10^3
1×10^{-3}	--+	+	±	±	-
2×10^{-3}	--+	+	±	±	-
4×10^{-3}	-	±	-	-	-
8×10^{-3}	-	±	-	-	-
16×10^{-3}	-	-	-	-	-
32×10^{-3}	-	-	-	-	-
32×10^{-4}	-	-	-	-	-

Similar results were obtained with both heat treated (60°C, 20') and non-treated samples. Only one set of experiments is presented.

Table 15 **Effect of Conjugated Antibody Concentration on the Residual Color Formation in the Supernatant, Using the Centrifugation Procedure.**

Conjugated Antibody	E. coli concentration /ml		
	10^7	10^5	10^3
2×10^{-4}	5.2	1.4	0.8
8×10^{-4}	0.6	0.9	0.0
32×10^{-4}	0.5	0.2	0.0

The values (activities) expressed in O.D. at 410 nm are the difference between blank and E. coli containing tests. Milk samples, containing varying concentrations of E. coli, were diluted with saline (1:10) and heated (60°C, 20 min). Conjugated antibody at different concentrations were added, incubated at 37°C for 150 min, and centrifuged at 11000 g for 20 min. ALP activity was determined in the supernatant and was subtracted from the activity in the blank.

Table 15 Effect of Antigen (E. coli) and Conjugated Antibodies Concentrations on Color Development of the Precipitate - In Saline

dilution of Ab-conj	E. coli concentration /ml				
	Blank	10^7	10^6	10^5	10^3
1×10^{-3}	±	++++	+++	+++	++
2×10^{-3}	±	++++	+++	+++	++
4×10^{-3}	±	++++	+++	+++	++
8×10^{-3}	-	++++	+++	+++	+
16×10^{-3}	-	++++	+++	+++	±
32×10^{-3}	-	++++	+++	++	±
32×10^{-4}	-	++++	++	-	-

Similar results were obtained with both heat treated (60°C, 20') and non-treated samples. Only one set of experiments is presented.

Table 17 **Effect of Milk Dilution With Saline on Color Development in the Precipitate using the Centrifugation Procedure with Different Concentrations of Antigen (E. coli) and Conjugated Antibodies.**

Milk dilution	conc. of conjugated Ab	blank	E. coli concentration /ml			
			10^7	10^6	10^5	10^3
1:2	20×10^{-3}	-	+++	++	-	-
	32×10^{-3}	-	+++	++	-	-
	32×10^{-4}	-	-	-	-	-
1:5	20×10^{-3}	-	++++	+++	+	±
	32×10^{-3}	-	++++	+++	+	±
	80×10^{-3}	-	++++	++	+	±
	32×10^{-4}	-	+	±	±	±
1:10	1×10^{-3}	-	++++	+++	+	±
	2×10^{-3}	-	++++	+++	+	±
	4×10^{-3}	-	++++	+++	+	±
	8×10^{-3}	-	++++	+++	+	+
	20×10^{-3}	-	++++	+++	+	+
	32×10^{-3}	-	++++	+++	+	+
	80×10^{-3}	-	++++	++	±	±
	320×10^{-3}	-	+++	+	±	±

Table 18 Ammonia Concentration in Milk from Normal and Mastitic Udder

Milk Sample	Ammonia concentration (μmol/l)
Healthy udder	387 ± 61
Pasteurized udder	349 ± 34
Mastitic (CMT = 1)	667 ± 96
Mastitic (CMT = 2)	985 ± 183
Mastitic (CMT = 3)	1896 ± 436
Mastitic (CMT = 4)	3947 ± 1092

X ± SD ; n = 5

Table 19 Ammonia Production in Milk in Relation to Bacterial Count

Bacterial count / ml	Ammonia concentration (µmol/l)
0	368 ± 36
10^4	676 ± 54
10^5	912 ± 89
10^6	1617 ± 123
10^7	2764 ± 313
10^8	5158 ± 719

Ammonia determination was done 15 minutes after the addition of the bacteria (E. coli) to pasteurized milk.

n = 6, X ± SD

Table 20 Ammonia Production in Milk During Improper Storage Conditions

Time (min)	Ammonia concentration (µmol/l)
0	340 ± 31
5	936 ± 68
10	1210 ± 83
15	1390 ± 91
60	1670 ± 108
120	1843 ± 112
180	2267 ± 149
360	2562 ± 173
1440	8431 ± 746

Ammonia was measured with a DT-60 Ektochem system. Dilutions were made with H_2O.

n = 6; X ± SD.

Table 21 **Enzyme Activities in Milk**

Enzyme (U/l)	Milk Samples		
	fresh-Normal	fresh-Mastitic	Pasteurized
CK	1	531	0
AST	7	680	0
ALT	0	58	0
ALP	760	299	0
LDH	120	3015	12
D.LDH	0	0	0
GGT	3539	3418	0
Amylase	0	54	0
AcP	5.2	10.6	0

Mean activity ; n = 6

Table 22 Milk Testing Using the "APIZYM" Kit

No	Enzyme Assay	A	B	C	D	E	F
1	Control	0	0	0	0	0	0
2	Phosphatase alcaline	0	5	5	5	5	5
3	Esterase (C 4)	0	1	1	1	0.5	1
4	Esterase Lipase (C 8)	0	1	1	1	1	1
5	Lipase (C 14)	0	0	0	0	0	0
6	Leucine arylamidase	0	5	1	1	1	1
7	Valine arylamidase	0	0	0.5	0	0	0
8	Cystine arylamidase	0	0	0.5	0	0	0.5
9	Trypsin	0	0	0	0	0	0.5
10	Chymotrypsin	0	0	0	0	0	0
11	Phosphatase acid	0	5	5	5	5	5
12	Naphthol-AS-BI-phosphohydrolase	1	5	5	5	5	5
13	α galactosidase	0	0	0	0	0	0
14	β galactosidase	0	1	0.5	0.5	0	1
15	β glucuronidase	0	3	1	5	5	5
16	α glucosidase	0	4	0.5	0.5	2	2
17	β glucosidase	0	0	0	0	0	0
18	N-acetyl-β glucosaminidase	0	5	1	1	5	2
19	α mannosidase	0	3	5	5	5	5
20	α fucosidase	0	0	0	0.5	1	1

A. Pasteurized milk
B. Pasteurized milk + staphylococcus (10^6/ml)
C. Pasteurized milk + micrococcus (10^6/ml)
D. Pasteurized milk + E. coli (10^6/ml)
E. Pasteurized milk + Strep. uberis (10^6/ml)
F. Fresh milk, no detectable pathogens = control

Table 23 Detection of Bacteria in Milk Using Nitrite Tests

Test	Color formation
a. Milk from healthy udder	-
b. Pasteurized milk	-
c. Pasteurized milk + NO_3 0 time	-
d. Pasteurized milk + NO_3 + E. coli*, 0 time	-
e. Pasteurized milk + NO_3, 24 hr, 37°C	++
f. Pasteurized milk + NO_3 + E. coli*, 90 min.,37°C	++
g. Mastitic milk	++
h. Mastitic milk, frozen 48 hr and thawed	++
i. Mastitic milk + NO_3 + sonicated E. coli* 90',37°C	++
j. Pasteurized milk + E. coli, 37°C, 90'	+

n = 12. *Similar results were obtained with both E. coli and S. aureus.
Rxn conditions: 5 ml milk; 0.1 ml 1% $NaNO_3$ incubation 37°C, 90 minutes.
At longer period of incubation, sterile conditions are required.

Table 24 **Leucocytes Detection in Milk By Esterase Activity, Using Urinalysis Strip**

Test	Color formation
Milk from healthy udder	-
Pasteurized milk	-
Mastitic milk, 3 x 10^2/ml E. coli	+++
Mastitic milk, E. coli following centrifugation	+
Heated (70°C, 10 min) mastitic milk	-

Strip was dipped into milk and read after 60 sec. Color developed was compared to the given color chart.

Table 25 Detection of Bacteria in Milk and Saline Using Tetrazolium

Test	Color formation
Normal milk	-
Mastitic milk	++++
Pasteurized milk	-
Pasteurized milk + E. coli	++++
Pasteurized milk + S. aureus	++++
Saline	-
Saline + E. coli	+++
Saline + S. aureus	+++
Milk + E. coli - freeze 24h, thawing	+
Milk + E. coli - super. following centrifugation	-
Milk or saline, + frozen bacteria	-

Test conditions as described for urine

Table 26 **Effect of incubation media, blocking of the reaction well and albumin concentration in tested samples on the reaction.**

Suspension	Blocking (casein)	BSA concentration (mg/ml)							
		0.3	0.6	1.25	2.5	5	10	20	40
Saline	-	0.810	0.885	0.892	0.812	0.716	0.532	0.495	0.242
	+	0.537	0.582	0.599	0.589	0.560	0.488	0.357	0.249
Milk	-	0.066	0.067	0.056	0.072	0.08[1]	0.057	0.108	0.151
	+	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006

mean of 3; a representing experiment.

incubation at 37°C, for 45 minutes.

conjugated antibody in a dilution of 1:200.

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