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THE DIAGNOSIS OF SUBCLINICAL MASTITIS IN LACTATING COWS: A COMPARISON OF CYTOLOGICAL METHODS AND A MONOVALENT RADIAL IMMUNODIFFUSION TEST

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ABSTRACT

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The diagnostic accuracy of conventional methods for the diagnosis of subclinical mastitis, such as the direct microscopic count (DMC) and electronic cell count (ECC) either used alone or in combination with bacteriological examinations, according to international standards, were compared with DNA determinations and a radial immunodiffusion test (MMT). The latter is based on single radial immunodiffusion of bovine serum albumin (BSA) in agarose containing anti-BSA serum.

This investigation was conducted on 1 008 foremilk samples, collected via the teat canal from 179 dairy cows, plus 56 samples obtained from 14 of them by means of cisternal puncture.

It is concluded that BSA concentrations in milk causing an MMT diameter $\geq$8mm are diagnostic for mastitis in lactating cows under the conditions existing in this experiment. The coefficient of variation of the MMT (14,67%) is significantly smaller than that of the DMC (87,78%), ECC (72,25%) and DNA content (37,19%) respectively and lies within the 15% limit recommended for diagnostic methods.

Diagnoses made by MMT, DMC and ECC over 3 successive days varied by 4,1%; 14,5% or 28,4% respectively. The reduced repeatability of the MMT resulted from genuine changes in udder health whereas 10,1% and 24,3% of variance observed for the DMC and ECC respectively were due to other factors.

In comparison to the MMT, mastitis diagnosis based on international standards resulted in 43,13$\pm$20,8% false positives. These are mainly due to teat canal infections simulating mastitis.

When both the MMT and international mastitis standards were used it was possible to distinguish between quarters with irrelevant and relevant teat canal infections, non-specific cellular reactions and septic or aseptic mastitis without having to resort to cisternal puncture.

Staphylococcal *beta* toxin inoculated into the teat canal, facilitated studies on the sequence of events leading to elevated BSA levels and cellular counts in the udder. A pre-inflammatory leucocytosis, resulting from passage of small amounts of toxin into the teat cistern was shown to occur in this investigation.

The diagnosis of subclinical mastitis in dry cows and within the first week *post partum* is as inaccurate by means of the MMT as by conventional methods.

INTRODUCTION

A previous investigation of various diagnostic methods (Giesecke, Van den Heever, Du Toit & Beyer, 1973), including a radial immunodiffusion test with a polyvalent antiserum produced from mastitic milk (Morris & Hobbs, 1971), casts serious doubts on the value of some existing methods, used for the diagnosis of subclinical mastitis in lactating, and, especially, dried-off dairy cows. Not only was there need for improved accuracy, but a method which would determine whether bacteria isolated from milk samples represented relevant infections of the udder or teat canal, or were irrelevant contaminants, was called for. Since polyvalency of the antiserum, used during the previous study (Giesecke *et al.*, 1973), was thought to be responsible for discrepancies between the results obtained with this technique, cytological methods and tests based on DNA levels, the various antigens involved were isolated. The antigen responsible for the most significant precipitin ring was identified as BSA (Viljoen, 1974).

It has been known for some time that BSA is present in cow's milk (Wells & Osbourne, 1921). Peskett (1932) briefly mentioned the identification of traces of BSA in milk by a precipitation test and suggested that levels of BSA might increase during secretion of abnormal milk. Using crystalline albumin isolated from milk by Polis, Shmukler & Custer (1950), Coulson & Stevens (1950) showed its antigenic identity to BSA. Lecce & Legates (1959) pointed out that the marked increase of this protein in the mastitic quarter approached an all-or-none phenomenon since it was usually impossible to detect it in milk sampled simultaneously from normal quarters of the same cow. The protein isolated from mastitic milk was identified electrophoretically as BSA by Shah, Morse & Pitkin (1963).

By means of an immunochemical method Dixon, Weigle & Vazquez (1961) detected 0,1–0,4 mg/ml of BSA in early milk, 0,1–0,2 mg/ml in true milk and 0,3–2,9 mg/ml in colostrum. However, the high colostral BSA concentrations gradually change to low lacteal concentrations within the first 1–5 days after partus (Larson & Kendall, 1957; Larson, 1958; Velten & Welz, 1961). Practically the same BSA concentations found by Dixon *et al.* (1961) in early milk and by Rolleri, Larson & Touchberry (1955) and Jenness, Larson, McMeekin, Swanson, Whitnah & Whitney (1956) from the 2nd to the 6th month of a lactation period, occur in normal quarters some 5–7 days after calving (Velten & Welz, 1961).

Fey (1960) and Nicolet (1962a, b) studied mastitic secretions using antisera against bovine serum. On immuno-electrophoresis it was found that more BSA and globulin appear in pathological secretions than in the normal milk. Increases in these proteins in mastitic milk were also found by Weigt (1959a), Velten & Welz (1961) and Shah *et al.* (1963). From his studies on experimental bacterial and aseptic mastitis induced by intramammary administration of staphylococci, streptococci, corynebacteria, *Escherichia coli* and non-specific irritants, Nicolet (1962a, b) concluded that in experimental *E. coli* mastitis, at any rate, the serum proteins had already started to appear two hours after artificial infection. Similar changes resulted from intramammary administration of *Brucella abortus* Strain 19 (Wisniowski, Grajewska, Romanuikowa, Grajewski & Drozdzynska, 1968) and sterile NaCl solution of various concentration

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(Grajewska, 1971). The close association between increased concentrations of BSA in milk and natural cases of mastitis due to *Staphylococcus aureus*, *Pseudomonas aeruginosa* and artificially induced streptococcal (*Streptococcus agalactiae*) or aseptic mastitis was confirmed by Shah & Morse (1964).

The specificity of elevated BSA levels as indicators of increased epithelial permeability in lactating mastitic mammary glands was first suggested by Lecce & Legates (1959). This viewpoint was supported by Drozdzynska (1971), who found that increased concentrations of a serum protein such as BSA in mastitic secretions occurred concurrently with a decrease of BSA in the blood of the affected animal. Furthermore, Mackenzie, Outteridge & Lascelles (1966), Mackenzie & Lascelles (1968) and Lascelles, Mackenzie & Outteridge (1971) showed that human serum albumin, administered parenterally to ewes infected artificially with *S. aureus*, increased markedly in concentration in the milk during the early stages of mastitis.

The specificity of elevated BSA levels in milk as indicators of mastitis is supported by classic studies on inflammation, which indicate that one of the earliest responses to an irritant is an increased topical capillary permeability of tissue (Landis, 1925/26). This leads *inter alia* to an outpouring of plasma proteins, especially serum albumin (Anderson, 1961) into the affected tissue.

The specificity of elevated BSA levels in milk as an indicator of epithelial irritation was implied by Dixon *et al.* (1961), who postulated that the cytoplasm of epithelial cells selectively transfers albumin and immunoglobulins. In the normal mammary gland these cells readily transmit gamma globulin and exclude, to a greater extent, the smaller albumin molecules. This situation is, however, distinctly altered by irritants entering the udder cavity (Lascelles *et al.*, 1971).

Elevated levels of serum albumin in milk coincide exactly with the acute clinical stages of mastitis (Schalm, Carroll & Jain, 1971). This can also be expected to apply to subclinical mastitis. The excellent graphical representation of the increased lacteal concentration of BSA during various stages of mastitis (Schalm *et al.*, 1971) also suggests that failure to record elevated BSA levels during chronic or acute mastitis respectively (Nilsson, 1958; Weigt, 1959b) could possibly be due to differences in the assay methods employed.

Available information suggests that quantitative BSA determinations on milk could serve as an objective, accurate method of diagnosing subclinical mastitis. Such determinations can be accurately made by means of the single radial immunodiffusion test, described by Mancini, Carbonara & Heremans (1965) for serum proteins and modified by Viljoen (1974) for BSA in milk. In the present investigation the diagnostic value of this test was compared with that of somatic cell counts, DNA concentrations and bacteriological examination.

Material and Methods

Experimental animals

The 177 Friesland-type and 2 Jersey cows used differed in age, number of lactations, daily milk yield and stage of lactation. These cows were free from tuberculosis and brucellosis, clinically healthy in other respects and in good condition. They were milked by machine.

Design of the experiment

Six series of trials were carried out:

SERIES I: 149 quarters of 38 cows were examined once to determine the interrelationship between udder health and BSA concentrations in milk at different stages of lactation.

SERIES II: 126 quarters of 32 cows were examined once to determine the interrelationship between BSA and international cytological and bacteriological standards for the diagnosis of subclinical mastitis in lactating cows at different stages of lactation.

SERIES III: 74 quarters of 19 cows were examined on 3 successive days to determine the daily fluctuations of BSA levels and somatic cell counts in milk.

The 89 cows studied in Series I to III were in a herd that was poorly managed.

SERIES IV: 301 quarters of 76 cows from an extremely well managed herd were examined once and the data obtained compared with those from Series I, II and III.

The subsequent experiments were performed on 12 Friesian and 2 Jersey cows of the Veterinary Research Institute, Onderstepoort.

SERIES V: 48 quarters of 12 cows were sampled conventionally on each of 3 successive days and on 1 occasion by cisternal puncture to determine the effect of teat canal infections on the bacteriological, cytological and immunochemical diagnosis of mastitis.

SERIES VI: Samples were collected conventionally from 28 quarters of 7 cows on each of 3 successive days (Day −1; Day 0; Day +1). After the morning milking of the 2nd day (Day 0) 0,1 ml of a sterile 60% suspension of staphylococcal *beta* toxin in 10% polyacrylamide gel was injected aseptically into the teat canals of the 2 right quarters (RF and RH) of each of 5 cows by means of sterile disposable plastic nozzles, 3 mm in length. The toxin was capable of lysing washed sheep erythrocytes at a dilution of 1:2 048.

The quantity of gel administered had a length of 5 mm if measured on a horizontal surface as compared to an average teat canal length of 10 mm (McDonald, 1968a, b; Appelman, 1969, 1970). The suspension remained in the teat canals for 5 hours and was flushed out during the afternoon milking. Conventional and cisternal samples were collected 17 hours later. Control values were obtained from 2 cows which received 0,1 ml of 10% polyacrylamide gel without toxin in the left front (LF) teat canals and experienced momentary insertion of the plastic nozzles in the right hind (RH) teat canals. The experiment served to determine the effect of slow release of staphylococcal toxin in the teat canal on the somatic cell count and BSA concentration in milk.

Sampling

After the usual premilking disinfection, the udders were thoroughly dried with disposable paper towels and the teats, especially the tips, vigorously swabbed with cotton wool moistened with 70% alcohol. In the lactating cows the first 3 jets of milk from each quarter were discarded and 20 ml of fore-milk sampled aseptically into sterile McCartney bottles. Puncture of teat cisterns, required for SERIES V and VI, was performed as described by Giesecke, Van den Heever, Hope & van Staden (1968) for gland cisterns.

In the case of dry cows, about 20 ml of secretion was collected conventionally after the first jet had been discarded.

Laboratory examinations of milk

A total of 1 008 samples was subjected to the following laboratory examinations immediately after sampling:

a. *The direct microscopic count* (DMC) was conducted in duplicate on whole milk smears processed by the standard methods of the American Public Health Association (Anon., 1967), using a calibrated platinum wire loop. The smears were stained by the Broadhurst-Paley method (Schalm, 1962). All clearly recognizable somatic cells were counted in 20 microscopic fields, randomly selected vertically and horizontally across the smears; 40% of the fields were in the central area of the smear and 60% in the marginal area.

b. *The electronic cell count* (ECC) was performed in duplicate with a Model B Coulter Counter equipped with a 70 μm aperture tube. The milk was processed for counting according to Tolle, Zeidler & Heeschen (1966) but using a Coulter Dual Diluter and a commercially available fat solvent*.

c. *Desoxyribonucleic acid* (μgDNA/ml of milk) was determined according to Dische (1930, cited by Chargaff & Davidson, 1955).

d. *The single radial immunodiffusion test for BSA*, herein referred to as the Monomastest (MMT), was performed as described by Morris & Hobbs (1971), but using a monovalent antiserum.

Different batches of antiserum were standardized against a solution containing 0,5 mg BSA/ml which caused a precipitin zone 7,5–8 mm in diameter with the original antiserum of Viljoen (1974). Discrepancies caused by weaker or stronger new antisera were compensated for by either adding to or subtracting the BSA values obtained with the batch of antiserum concerned from the standard, a method authenticated by the statistical data provided by Mancini *et al.* (1965).

After inoculation the MMT plates were left for 24 hours at room temperature (24–28°C) before measuring the diameter of the precipitin ring by means of graduated callipers.

e. *Bacteriological examination* of milk samples was performed as described by Giesecke, Nel & Van den Heever (1968).

Subclinical mastitis in lactating cows was diagnosed according to cytological and bacteriological standards suggested by the International Dairy Federation (Kästli, 1967; Tolle, 1971) and reviewed in detail by Giesecke & Van den Heever (1974)

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Colostrum was regarded as being positive for mastitis if bacterial growth occurred and on the presence of macroscopic and/or microscopic pus floccules.

Results and Discussion

SERIES I: Interrelationship between Udder Health and BSA Concentrations at Different Stages of Lactation

The diameters of precipitin rings as a measure of the BSA concentrations of milk, obtained from 149 quarters of 38 cows at different stages of lactation, are illustrated in Fig. 1.

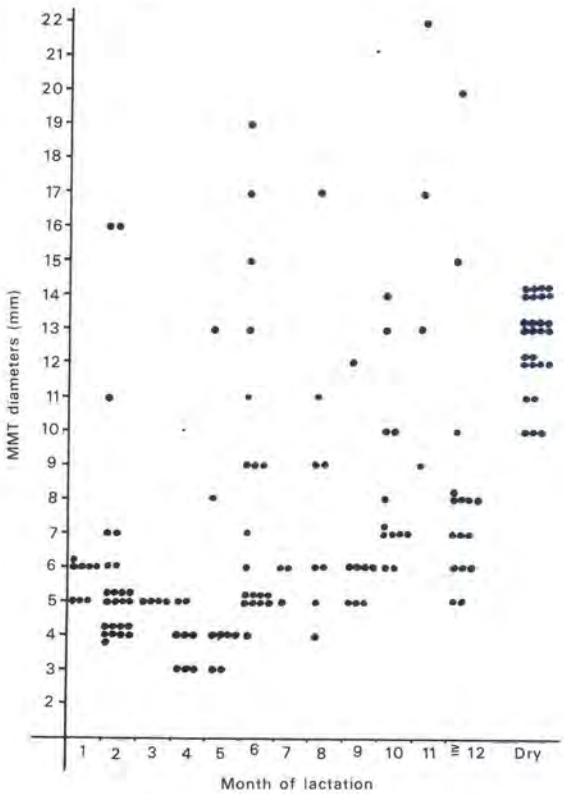

FIG. 1 BSA concentrations, expressed as diameter of the precipitin ring, in 149 quarter samples obtained from 38 cows at different stages of lactation
● = 1 quarter

These data suggest a considerable scattering of BSA concentrations in these udder secretions. However, an accumulation of data is recognizable along the bottom fringe of the diagram. Since BSA concentrations in normal and mastitic milk are markedly different (Larson & Kendall, 1957; Dixon *et al.*, 1961; Velton & Welz, 1961; Schalm *et al.*, 1971), it was considered essential to establish a base-line for the distinction of mastitis negative from other udder secretions in order to determine normal lactational changes of BSA in milk.

Mastitis negative quarters

For the purposes of this investigation, quarters were considered to be mastitis negative when their milk was free from pathogenic micro-organisms and they had a somatic cell count $<500\times10^3$ cells/ml, irrespective of whether the count was obtained by

TABLE 1 $\bar{X}$, SD and CV of results obtained from 81 mastitis negative quarters examined by MMT, DMC, ECC and for DNA

Criteria		Month of lactation												
		1	2	3	4	5	6	7	8	9	10	11	≧12	Dry
MMT diameters (mm)	$\bar{X}$	5,0	4,63	4,0	3,88	3,67	5,16	No	5,25	5,57	6,86	No	7,43	12,67
	SD	0,0	0,67	0,0	0,83	0,52	0,41	sample	0,96	0,54	0,69	sample	0,79	1,33
	CV	0,0	14,54	0,0	21,54	14,08	7,90		18,24	9,59	10,06		10,59	10,49
DMC ($\times 10^3$ cells/ml)	$\bar{X}$	250	122	83	119	125	167	No	75	221	1 276	No	314	4 022
	SD	0,0	88	29	800	184	93	sample	50	95	1 406	sample	193	2 609
	CV	0,0	71,42	34,64	67,28	146,97	55,86		66,67	42,96	110,23		61,42	64,86
ECC ($\times 10^3$ cells/ml)	$\bar{X}$	490	523	461	554	262	478	No	595	547	866	No	720	19 061
	SD	14	414	94	265	183	349	sample	243	233	502	sample	648	24 184
	CV	2,88	79,06	20,37	47,77	70,03	73,03		40,89	42,52	57,95		89,96	126,88
DNA (μg/ml)	$\bar{X}$	460	560	420	485	783	632	No	675	796	1 429	No	1 973	2 432
	SD	56,56	234,88	58,88	145,70	403,32	364,99	sample	210,00	523,25	920,08	sample	897,90	1 538,40
	CV	12,29	41,88	14,02	30,04	51,49	57,78		31,11	65,76	64,41		45,51	63,25

DMC and/or ECC. BSA concentrations, expressed as diameters of precipitin rings, of the 81 mastitis negative quarters selected thus are illustrated in Fig. 2, and mean values ($\overline{X}$), standard deviation (SD) and coefficient of variation (CV) of results obtained by MMT, DMC, ECC and for DNA are presented in Table 1.

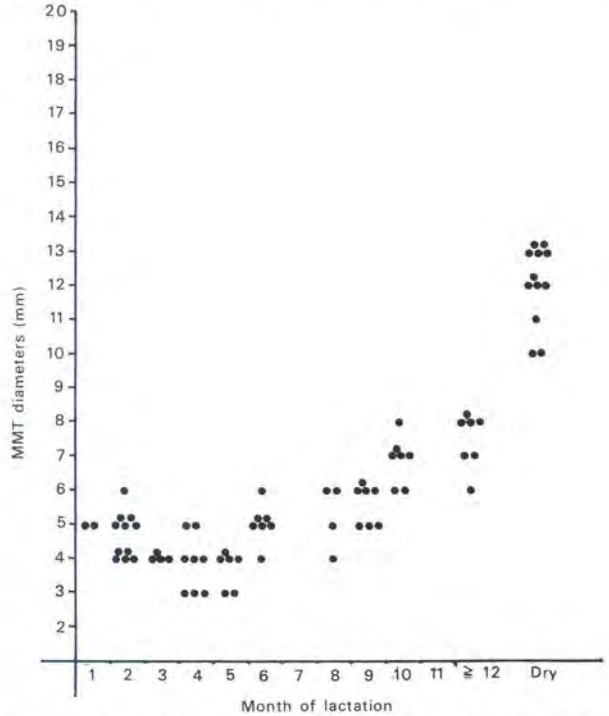

FIG. 2 BSA concentrations, expressed as diameters of the precipitin rings, in 81 mastitis negative samples obtained from 38 cows at different stages of lactation
● = 1 quarter

The mean values and standard deviations of BSA concentrations in the mastitis negative milk of cows at different stages of lactation are depicted in Fig. 3. The corresponding values of DMC, ECC and DNA determinations are given in Fig. 3a, 3b and 3c. The graphs illustrate the pattern of events during the course of a normal lactation.

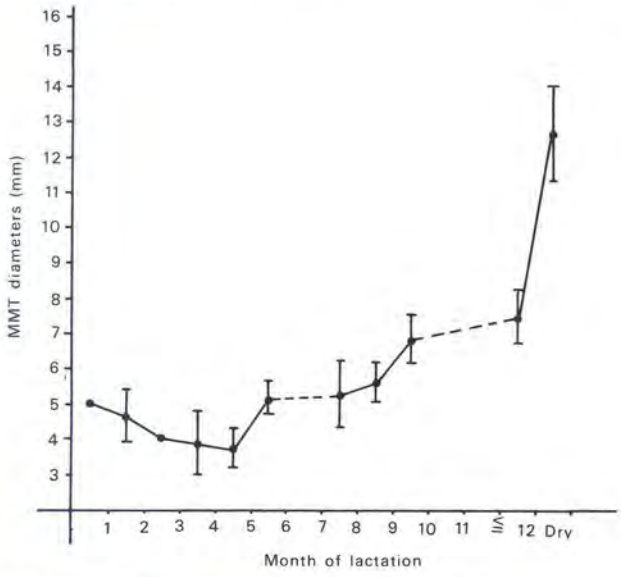

FIG. 3 Mean and standard deviation of BSA concentrations in 81 mastitis negative samples, expressed as diameters of precipitin rings
— — — — = presumed values since no data available

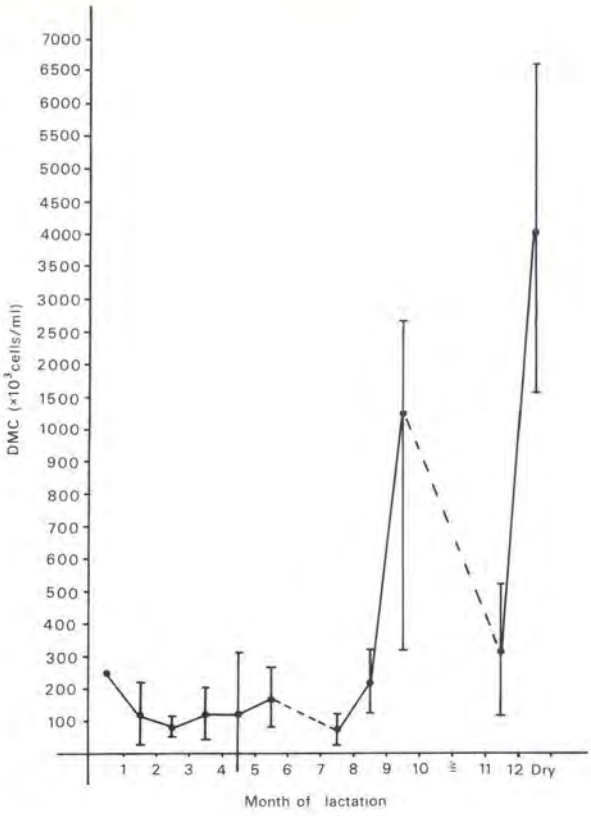

FIG. 3a Mean and standard deviation of DMC in 81 mastitis negative samples
— — — — = presumed values since no data available

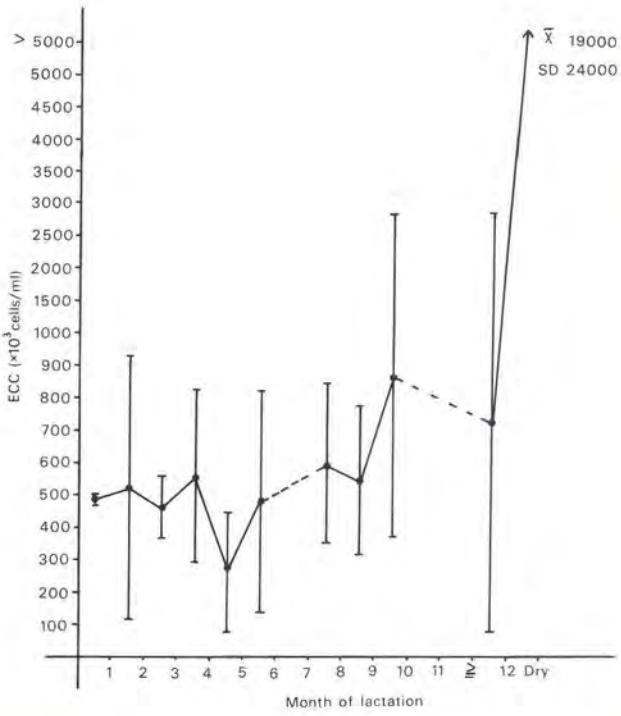

FIG. 3b Mean and standard deviation of ECC in 81 mastitis negative samples
— — — — = presumed values since no data available

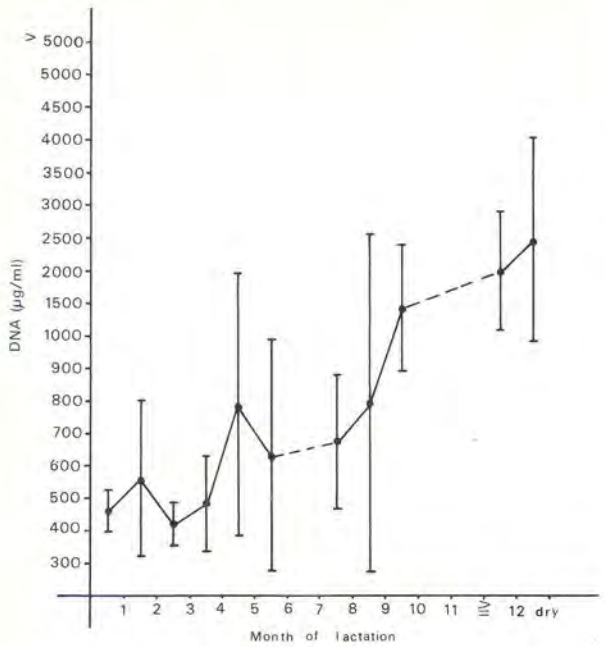

FIG. 3c Mean and standard deviation of DNA concentrations in 81 mastitis negative samples
— — — — = presumed values since no data available

If a normal lactation period is regarded as being 10 months, it appears justifiable to suggest an MMT standard for mastitis negative lactating cows as shown in Fig. 4.

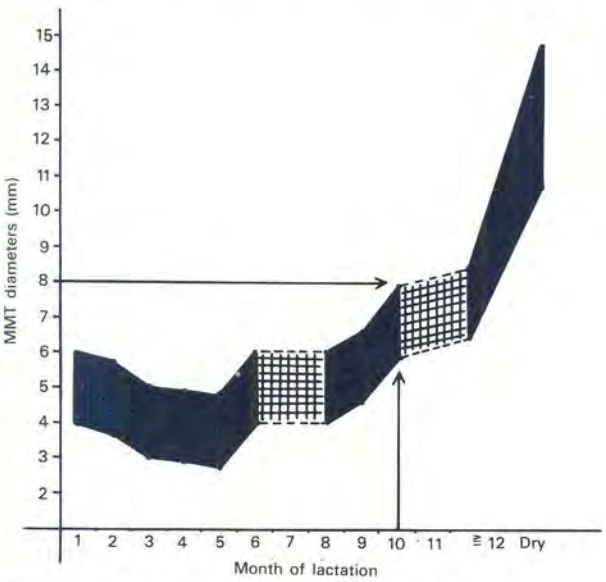

FIG. 4 The MMT standard suggested for mastitis negative lactating cows with lactation periods of 10 months
— — — — = presumed values since no data available

The suggested standard can only be applied if the stage of lactation of the cows to be examined is known. Since the mean diameter and standard deviation obtained from 56 MMT readings on mastitis negative milk from cows within a 10-month lactation period was 4,9 $\pm$ 2,5 mm and the maximum value was 7,55mm (Table 1 and Fig. 2), it is suggested that a MMT reading of below 8 mm indicates mastitis negative and 8 mm or above, mastitis positive milk. In view of the actual BSA concentrations (7,55 mm) present in milk obtained from mastitis negative cows in the 10th month of lactation (Fig. 2), the cricita MMT diameter of 8 mm is suggested instead of 7,4 mm, resulting from the standard deviation of the mean diameter (4,9 $\pm$ 2,5 mm).

Mastitis positive quarters

In this investigation the group of 59 quarters classified as "mastitis positive" consisted of 2 quarters that secreted milk containing pathogenic bacteria and $<500\times10^3$ cells/ml (latent infections), and 57 quarters secreting milk with pathogenic bacteria and $>500\times10^3$ cells/ml (subclinical mastitis). Nine quarters from dry cows were also classified as "mastitis positive" according to the previously mentioned criteria.

The BSA concentration of all 68 "mastitis positive" quarters, expressed as diameters of precipitin rings, are illustrated in Fig. 5; the mean values ($\overline{X}$), standard deviations (SD) and coefficient of variation (CV) of results obtained by MMT, DMC, ECC and for DNA are presented in Table 2.

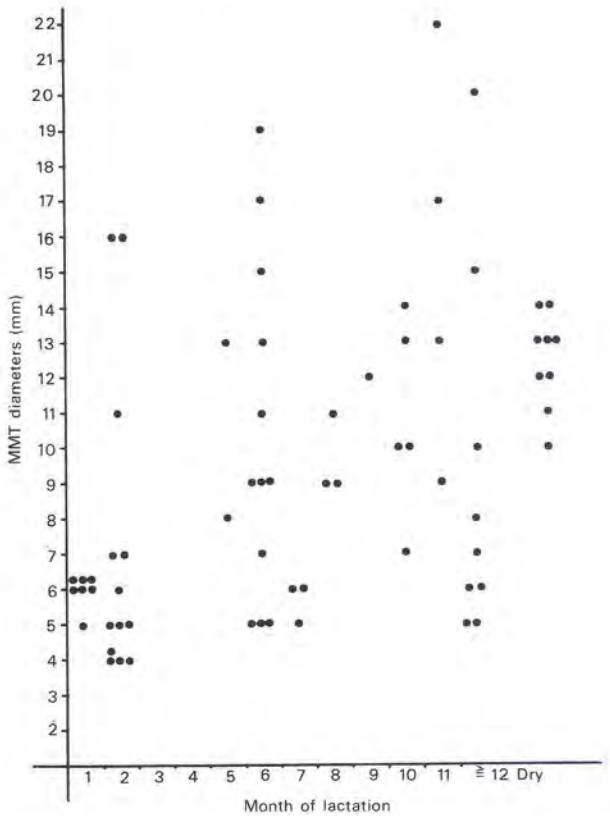

FIG. 5 BSA concentrations, expressed as diameters of precipitin rings, in 68 "mastitis positive" milk samples obtained from 38 cows at different stages of lactation
● = 1 quarter

The mean values and standard deviations of BSA concentrations in these 68 samples from cows in various stages of lactation can be taken to represent the abnormal pattern of BSA concentrations in udder secretions as illustrated in Fig. 6. There is considerable overlap between these values and those obtained with negative samples.

TABLE 2 $\bar{X}$, SD and CV of results obtained from 68 "mastitis positive" quarters examined by MMT, DMC, ECC and for DNA

Criteria		Month of lactation												
		1	2	3	4	5	6	7	8	9	10	11	≧12	Dry
MMT diameters (mm)	$\bar{X}$	5,83	7,23	No	No	10,50	10,33	5,67	11,50	12,00	10,80	15,25	9,11	12,44
	SD	0,41	4,34	sample	sample	3,54	4,77	0,58	3,79	0,00	2,78	5,56	5,16	1,33
	CV	6,99	60,06			33,67	46,20	10,19	32,92	0,00	25,69	36,46	56,62	10,71
DMC ($\times 10^3$ cells/ml)	$\bar{X}$	1 683	4 002	No	No	5 850	5 496	2 483	9 212	uncount-	6 450	8 638	3 050	6 062
	SD	1 619	5 105	sample	sample	5 728	6 706	369	927	able	3 884	7 738	2 229	2 347
	CV	96,19	127,55			97,91	122,02	14,84	10,06		60,21	89,59	73,08	38,71
ECC ($\times 10^3$ cells/ml)	$\bar{X}$	2 266	5 259	No	No	2 371	6 655	1 240	13 073	15 769	13 185	6 218	4 994	22 078
	SD	1 586	6 882	sample	sample	2 005	8 675	926	7 535	0,0	13 794	6 007	4 320	19 538
	CV	69,99	130,86			84,58	130,36	74,65	57,63	0,0	104,62	96,62	86,50	88,49
DNA (μg/ml)	$\bar{X}$	460	1 246	No	No	1 700	1 242	783	535	1 040	594	1 850	2 648	2 289
	SD	76,94	1 181,20	sample	sample	70,71	1 102,44	600,69	138,92	0,0	117,81	768,11	2 066,65	1 151,41
	CV	16,73	94,79			4,16	88,79	76,68	25,97	0,0	19,83	41,52	78,05	50,31

Whereas there can be little doubt about the close association between increased BSA concentrations in mature milk and inflammatory secretional disturbances of the udder epithelium (i.e. true mastitis), there is, however, good reason to believe that teat canal infections are mainly responsible for incorrect diagnoses of mastitis if the abovementioned cytological and bacteriological criteria are used (Birkner, 1964; Heidrich, Grossklaus & Mülling, 1964; Heidrich, Mülling & Birkner, 1964; Forbes, 1968a, b; Forbes & Herbert, 1968; Giesecke, Van den Heever, Hope & Van Staden, 1968; Forbes, 1969; Forbes, 1970a, b). It was therefore decided to employ a combination of immunochemical, cytological and bacteriological methods to distinguish between true mastitis, teat canal infections and milk from completely healthy udders. The key used for this purpose is shown in Table 3.

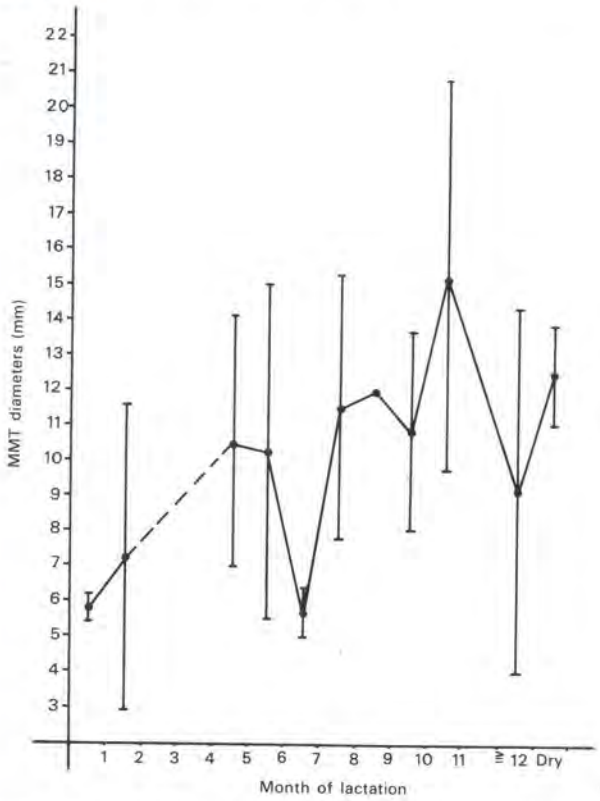

FIG. 6 Mean and standard deviation of BSA concentrations in 68 "mastitis positive" samples
— — — — = presumed values since no data availabie

The data from 68 "mastitis positive" quarters (Table 2 and Fig. 6) were reclassified on the basis of the abovementioned key. Particulars of quarters with either teat canal infections or true mastitis are given in Tables 4a, 4b and Fig. 7.

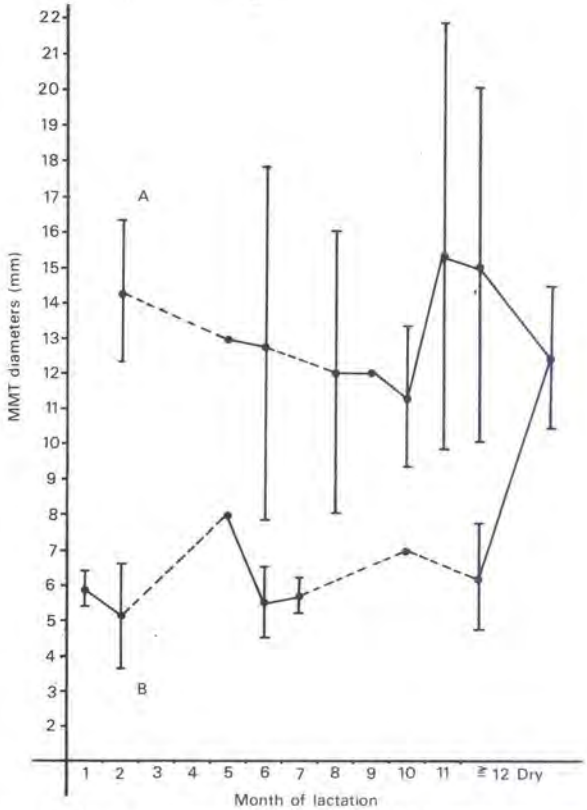

FIG. 7 Mean and standard deviation of BSA concentrations, expressed as diameters of precipitin rings, in 68 "mastitis positive" samples following the differentiation between true mastitis and teat canal infections
— — — — = presumed values since no data available
A = true mastitis B = teat canal infections

Thirty-one (52,7%) of the 59 "mastitis positive" quarters from cows in lactation for 12 months or less qualified for teat canal infections whereas the remaining 28 (47,5%) had true mastitis. If the reclassification was limited to the 46 "mastitis positive" quarters of cows that were in milk for 10 months or less, i.e. the normal period of lactation, 25 (54,4%) were classified as having teat canal infections whereas the remaining 21 (45,9%) were regarded as truly mastitic. Conventional cytological and bacteriological examination for mastitis performed on cows in normal lactation would therefore appear to produce 54,4% false positive diagnoses.

TABEL 3 A key for differentiation between mastitis and teat canal infections in lactating cows by means of a combination of immunochemical, cytological and bacteriological tests

MMT (mm)	Somatic cells/ml	Bacteriological culture	Mastitis diagnosis	Final diagnosis
<8.0	$<500\times10^3$	Negative	Negative	Negative
	$<500\times10^3$	Positive		Irrelevant* teat canal infection
	$>500\times10^3$	Positive		Relevant** teat canal infection
	$>500\times10^3$	Negative		Non-specific cellular reaction
≧8.0	$\geqq500\times10^3$	Negative	Positive	Aseptic mastitis
	$\geqq500\times10^3$	Positive		Septic mastitis

* Irrelevant due to lack of a cellular reaction; it includes extraneous contamination of completely normal milk
** Relevant since infection is severe enough to elicit a cellular reaction in the udder cavity, suggesting an ascending mastitogenic tendency

TABLE 4a $\bar{X}$, SD and CV of MMT, DMC, ECC and DNA in 30 lactating quarters with teat canal infections and 9 mastitic dry quarters

Criteria		Month of lactation												
		1	2	3	4	5	6	7	8	9	10	11	≧12	Dry
MMT diameters (mm)	$\bar{X}$	5,83	5,10	No	No	8,0	5,50	5,67	No	No	7,0	No	5,80	12,44
	SD	0,41	1,20	sample	sample	0,0	1,0	0,58	sample	sample	0,0	sample	0,84	1,33
	CV	6,99	23,47			0,0	18,18	10,19			0,0		14,43	10,71
DMC ($\times 10^3$ cells/ml)	$\bar{X}$	1 683	1 563	No	No	1 800	825	2 483	No	No	1 750	No	1 570	6 062
	SD	1 619	834	sample	sample	0,0	156	369	sample	sample	0,0	sample	719	2 347
	CV	96,19	53,39			0,0	18,84	14,84			0,0		45,80	38,71
ECC ($\times 10^3$ cells/ml)	$\bar{X}$	2 266	2 657	No	No	953	607	1 240	No	No	1 078	No	3 649	22 078
	SD	1 586	846	sample	sample	0,0	408	926	sample	sample	0,0	sample	1 695	19 553
	CV	69,99	31,84			0,0	67,20	74,65			0,0		46,44	88,49
DNA (μg/ml)	$\bar{X}$	460	820	No	No	1 750	520	783	No	No	560	No	1 826	2 289
	SD	76,94	435,43	sample	sample	0,0	80,0	600,69	sample	sample	0,0	sample	1 351,25	1 151,41
	CV	16,73	53,10			0,0	15,39	76,68			0,0		74,00	50,31

TABLE 4b $\bar{X}$, SD and CV of MMT, DMC and DNA in 29 truly mastitic lactating quarters

Criteria		Month of lactation											
		1	2	3	4	5	6	7	8	9	10	11	≧12
MMT diameters (mm)	$\bar{X}$	No	14,33	No	No	13,0	12,75	No	11,50	12,0	11,75	15,25	13,25
	SD	sample	2,89	sample	sample	0,0	3,92	sample	3,79	0,0	2,06	5,56	5,38
	CV		20,14			0,0	30,74		32,92	0,0	17,55	36,46	40,58
DMC ($\times 10^3$ cells/ml)	$\bar{X}$	No	12 133	No	No	9 900	7 831	No	9 212	9 900	7 625	8 638	4 900
	SD	sample	4 931	sample	sample	0,0	7 208	sample	927	0,0	3 303	7 738	2 085
	CV		40,64			0,0	92,05		10,06	0,0	43,32	89,59	42,54
ECC ($\times 10^3$ cells/ml)	$\bar{X}$	No	13 931	No	No	3 789	9 679	No	13 073	31 537	16 212	6 218	6 676
	SD	sample	11 588	sample	sample	0,0	9 317	sample	7 535	0,0	13 879	6 007	6 257
	CV		83,18			0,0	96,28		57,63	0,0	85,61	96,62	93,73
DNA (μg/ml)	$\bar{X}$	No	2 666,67	No	No	1 650	1 603	No	535	1 040	603	1 850	3 675
	SD	sample	1 892,97	sample	sample	0,0	1 208,61	sample	138,92	0,0	134,26	768,11	2 534,27
	CV		70,99			0,0	75,42		25,97	0,0	22,28	41,52	68,27

Fig. 7 indicates that diagnostic errors due to the wide range of BSA concentrations in milk from quarters with mastitis or teat canal infections may also occur with use of the MMT if it gives a reading of 8 mm as demonstrated by the precipitin rings with a diameter of 7 and 8 mm, illustrated in Fig. 8.

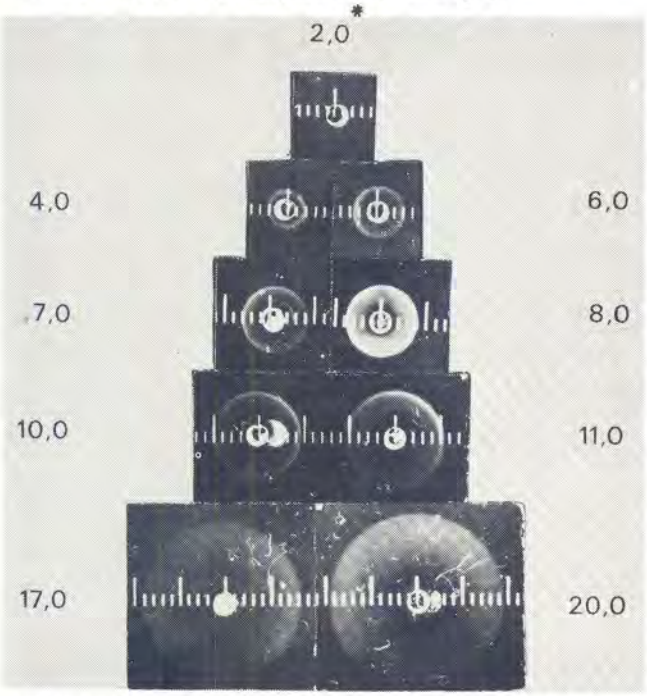

FIG. 8 Monovalent radial immuno-diffusion reactions
*diameter of precipitation zone in mm

Comment

It is apparent from the data illustrated in Fig. 3, 7 and 8 that MMT precipitation zone diameters of $<7,5$ and $>8,5$ mm respectively indicate the absence or presence of mastitis. Diameters between 7,5 mm and 8,5 mm, however, have to be regarded as doubtful and such results were obtained in 4,7 %of the 149 lactating quarters examined. If additional cytological and bacteriological data are also taken into consideration the incidence of doubtful cases, diagnosed purely on MMT results, could be reduced from 4,7% to 2,9%. These figures are very satisfactory if compared with the abovementioned 54,4% false positive mastitis diagnoses provided by conventional methods.

Since no difference was seen between BSA concentrations of mastitis negative and positive udder secretions of dry cows (Fig. 2 and 7), the accurate diagnosis of subclinical mastitis in dried-off cows remains a problem. The MMT is obviously as unsuitable as the conventional tests for the diagnosis of sub-clinical mastitis in udder secretions collected at drying-off or during the dry period.

SERIES II: The Interrelationship between BSA Concentrations in Milk and International Standards used for the Diagnosis of Subclinical Mastitis in Cows at Different Stages of Lactation

A more detailed investigation appeared to be desirable particularly with regard to the cytological classification suggested by the International Dairy Federation (Kästli, 1967; Tolle, 1971) and the bacteria isolated from the milk samples.

The results obtained after examining 126 quarter samples from 32 lactating cows by means of the MMT, DMC, ECC and DNA determinations are summarized in Table 5. As in the case of SERIES I, the classification into mastitis negative and positive quarters is based on international standards, 500×10^3 cells/ml being regarded as the critical threshold. Latent infection, as defined by Kästli (1967) and Tolle (1971) and included in the "mastitis positive" group, was diagnosed in 6 quarters.

TABLE 5 A summary of data obtained from 32 lactating cows by means of MMT, DMC, ECC and DNA determinations

Mastitis diagnoses		Quarters					
		Mastitis negative			"Mastitis positive"		
Stage of lactation months		Early 1–3	Middle 4–6	Late 7–10	Early 1–3	Middle 4–6	Late 7–10
MMT diameters (mm)	3	1	1				
	4	26	16	3	5	4	1
	5	24	5	3	3	3	1
	6			4		2	8
	7			1		3	3
	8						1
	9					1	
	10						4
	11						
	12						1
	13						2
MMT diameters (mm)	$\bar{X}$	4,45	4,18	5,20	4,33	5,61	7,95
	SD	0,61	0,50	1,03	0,50	1,55	2,63
	CV	13,71	11,98	19,86	11,63	27,72	33,14
DMC ($\times 10^3$ cells/ml)	$\bar{X}$	67	73	100	2 178	1 792	4 044
	SD	55	55	97	1 851	1 441	3 814
	CV	81,70	75,69	97,18	84,98	80,38	94,33
ECC ($\times 10^3$ cells/ml)	$\bar{X}$	99	224	212	553	691	1 456
	SD	108	144	117	370	532	1 263,15
	CV	109,95	64,15	54,97	66,81	77,02	86,77
DNA (μg/ml)	$\bar{X}$	590	646	838	528	1 000	1 456
	SD	204,19	207,95	393,92	163,99	517,99	1 263,15
	CV	34,59	32,17	47,00	31,07	51,79	86,77

TABLE 6 Summary of cytological, bacteriological and immunochemical data concerning "positive" mastitis diagnoses of quarters in early lactation (1 to 3 months)

Bacterial isolates		Quarters with somatic cell counts of											
		$<300\times10^3$ cells/ml				300×10^3 to 500×10^3 cells/ml				$>500\times10^3$ cells/ml			
		S. aureus	*S. epidermidis*	*Str. agalactiae*	Aseptic	*S. aureus*	*S. epidermidis*	*Str. agalactiae*	Aseptic	*S. aureus*	*S. epidermidis*	*Str. agalactiae*	Aseptic
MMT diameters (mm)	4	—	1	—	—	—	—	—	—	3	—	—	1
	5	—	1	—	—	—	—	—	—	1	1	—	—
MMT diameters (mm)	$\bar{X}$		4,33				—				4,33		
	SD		0,57				—				0,51		
	CV		13,32				—				11,91		
DMC ($\times10^3$ cells/ml)	$\bar{X}$		150				—				3 192		
	SD		100				—				1 333		
	CV		66,66				—				41,75		
ECC ($\times10^3$ cells/ml)	$\bar{X}$		239				—				710		
	SD		152				—				347		
	CV		63,55				—				48,92		
DNA (μg/ml)	$\bar{X}$		560				—				512		
	SD		204,20				—				159,42		
	CV		36,46				—				31,15		

TABLE 7 Summary of cytological, bacteriological and immunochemical data concerning "positive" mastitis diagnoses of quarter in middle lactation (4 to 6 months)

Bacterial isolates		Quarters with somatic cell counts of											
		$<300\times10^3$ cells/ml				300×10^3 to 500×10^3 cells/ml				$>500\times10^3$ cells/ml			
		S. aureus	*S. epidermidis*	*Str. agalactiae*	Aseptic	*S. aureus*	*S. epidermidis*	*Str. agalactiae*	Aseptic	*S. aureus*	*S. epidermidis*	*Str. agalactiae*	Aseptic
MMT diameters (mm)	4	1	—	—	—	—	—	1	—	—	—	1	1
	5	—	—	—	—	—	—	—	—	2	—	—	1
	6	—	—	—	—	—	—	—	—	—	—	1	1
	7	—	—	—	—	—	—	—	—	—	1	1	1
	9	—	—	—	—	—	—	—	—	1	—	—	—
MMT diameters (mm)	$\bar{X}$			4,0				4,0				5,90	
	SD			0,0				0,0				1,51	
	CV			0,0				0,0				25,61	
DMC ($\times10^3$ cells/ml)	$\bar{X}$			50				500				2 069	
	SD			0,0				0,0				1 391	
	CV			0,0				0,0				67,22	
ECC ($\times10^3$ cells/ml)	$\bar{X}$			163				112				791	
	SD			0,0				0,0				517	
	CV			0,0				0,0				65,34	
DNA (μg/ml)	$\bar{X}$			590				600				1 074	
	SD			0,0				0,0				532,17	
	CV			0,0				0,0				49,56	

TABLE 8 Summary of cytological, bacteriological and immunochemical data concerning "positive" mastitis diagnoses of quarters in late lactation (7 months and longer)

Bacterial isolates		Quarters with somatic cell counts of											
		$<300\times10^3$ cells/ml				300×10^3 to 500×10^3 cells/ml				$>500\times10^3$ cells/ml			
		S. aureus	*S. epidermidis*	*Str. agalactiae*	Aseptic	*S. aureus*	*S. epidermidis*	*Str. agalactiae*	Aseptic	*S. aureus*	*S. epidermidis*	*Str. agalactiae*	Aseptic
MMT diameters (mm)	4	—	1	—	—	—	—	—	—	—	—	—	—
	5	—	—	—	—	—	—	—	—	1	—	—	—
	6	—	—	—	—	—	1	1	—	—	—	2	4
	7	—	—	—	—	—	—	—	—	1	2	—	—
	8	—	—	—	—	—	—	—	—	—	—	1	—
	10	—	—	—	—	—	—	—	—	3	—	—	1
	12	—	—	—	—	—	—	—	—	1	—	—	—
	13	—	—	—	—	—	—	—	—	1	—	1	—
MMT diameters (mm)	$\bar{X}$		4,0				6,0				8,22		
	SD		0,0				0,0				2,62		
	CV		0,0				0,0				31,92		
DMC ($\times10^3$ cells/ml)	$\bar{X}$		200				350				4 666		
	SD		0,0				71				3 774		
	CV		0,0				20,20				80,87		
ECC ($\times10^3$ cells/ml)	$\bar{X}$		109				196				2 668		
	SD		0,0				77				3 415		
	CV		0,0				39,42				161,75		
DNA (μg/ml)	$\bar{X}$		720				695				1 582		
	SD		0,0				360,62				1 325,62		
	CV		0,0				51,88				83,78		

The MMT, DMC, ECC and DNA data were in close agreement with those listed in Tables 1 and 2. The BSA values were also similar to those obtained previously (Fig. 3 to 6).

The "mastitis positive" quarters were further subdivided according to the lactational stage and the DMC values. With due consideration of the fact that critical cell count thresholds of either 300×10^3 or 500×10^3 cells/ml of milk are used for the diagnosis of subclinical mastitis in different countries (Kästli, 1967), the cell counts obtained from the "mastitis positive" samples were subdivided into those with $<300 \times 10^3$, between 300×10^3 to 500×10^3 and $>500 \times 10^3$ cells/ml.

The immunochemical, cytological and bacteriological data obtained for "mastitis positive" samples from cows in early, middle and late lactation are summarized in Tables 6, 7 and 8 respectively.

Data in Table 6, resulting from MMT, DMC, ECC and DNA determination on samples with $< 300 \times 10^3$ cells/ml milk, fall within the range of normal values previously established for milk drawn during the first 3 months of lactation from mastitis negative quarters (Fig. 2; Table 1). Two quarters were bacteriologically positive; bacterial growth for both was recorded as 2 plus, i.e. 10 to 20 colonies of *Staphylococcus epidermidis*. Since the sensitivity of the MMT with regard to the number of infecting micro-organisms required to elicit an increase of BSA concentrations in milk has not been determined, it is possible that some latent udder infections may not be detected, although it seems unlikely that the MMT would fail to show a reaction to infective organisms present in the udder lumen. Based on the key provided in Table 3, the micro-organisms isolated from samples with $>500 \times 10^3$ cells/ml milk and bacterial infection are considered to be from mastitis negative quarters with relevant teat canal infections.

On the same basis the data summarized in Table 7 suggest 1 irrelevant contamination, 1 truly mastitic quarter and either 11 or 10 cases of relevant teat canal infections, depending on whether the threshold is taken as $>300 \times 10^3$ cells/ml or $>500 \times 10^3$ cells/ml respectively.

The data summarized in Table 8 likewise suggest 1 irrelevant contamination and 13 or 12 relevant teat canal infections. Eight truly mastitic quarters were diagnosed, including the dubious case with *Streptococcus agalactiae* infection and a MMT diameter of 8 mm. Classification of this case as one of true mastitis seemed justified since DMC, ECC and DNA values were $1\,850 \times 10^3$ cells/ml, $3\,750 \times 10^3$ cells/ml and 1 940 μg/ml respectively together with a bacterial growth of 1 plus (< 10 colonies).

The investigation showed that 84 quarters were mastitis negative and 42 quarters "mastitis positive" (Table 5) if a somatic cell count of $>500 \times 10^3$ cells/ml was considered to be indicative of mastitis and if quarters with "latent infections" were included in the group of "mastitis positive" quarters. On further analysis of the "mastitis positive" quarters (Tables 6, 7 and 8) either 4 or 7 quarters show "latent" infection depending on whether a critical threshold of $>300 \times 10^3$ cells/ml or $>500 \times 10^3$ cells/ml is applied for non-latency. The remaining 35 to 38 quarters are mastitis positive according to international standards.

Contrary to this the MMT, combined with conventional cytological and bacteriological examinations, suggests 4 irrelevant contaminations, 19 quarters with relevant, i.e. potentially serious teat canal infections and only 9 truly mastitic quarters. Nine of 10 quarters conventionally diagnosed as having "aseptic mastitis" (>500 $\times$ 10^3 cells/ml minus bacteria) are shown by the MMT to exhibit increased somatic cell counts due to unknown reasons not associated with udder inflammation, viz. mastitis.

Application of international standards for mastitis plus the key in Table 3, to the 42 "mastitis positive" quarters results in the spectrum of diagnoses outlined in Table 9.

It is apparent that, according to international standards, there were 35 to 38 cases of mastitis (mean 86,9%) amongst the 42 "mastitis positive" quarters, representing latent infections and cases of subclinical mastitis diagnosed by means of a 300 or 500 $\times$ 10^3 cells/ml critical threshold. Nine (21,4% cases of mastitis were diagnosed by the MMT used according to the key of Table 3. Hence it would appear that 65,5% of quarters diagnosed as mastitis positive by conventional standards were in fact not mastitic.

If considered in terms of all 126 quarters examined, the diagnostic error is proportionally smaller but the actual magnitude remains unaltered. Thus of 126 quarters, a mean of 28,5% were mastitis positive by international standards whereas the MMT showed 7,1% as cases of true mastitis, suggesting that 21,5% of all quarters diagnosed as mastitis positive by international standards were in fact not mastitic.

Statistical analysis

In order to obtain more information on the diagnostic value of these methods tested, data from SERIES II were processed statistically.

The coefficients of variation of MMT, DMC, ECC and DNA determination are summarized in Table 10. The mean coefficients of variation for the MMT, DMC, ECC and DNA determinations of the mastitis negative samples are 14,67%; 87,78%; 75,25%, and 37,19% respectively. The magnitude of the variations measured is indicated by the corresponding standard deviations, which were 0,70 mm, 81 $\times$ 10^3 cells/ml, 121 $\times$ 10^3 cells/ml and 263,43 μg/ml for the MMT, DMC, ECC and DNA determinations respectively. Since the coefficient of variation of a diagnostic method should not exceed 15% on examination of normal specimens (Snedecor, 1961; Rayner, 1969) the MMT, performed on the 84 samples, is seen to possess a reproduceability which is markedly superior to that of DMC, ECC and DNA determinations.

TABLE 9 Break-down of 42 "mastitis positive" quarters according to: A. The key in Table 3
B. International dairy federation standards only

	Diagnostic criteria	"Mastitis positive" quarters (%=percentage of 42 quarters concerned)						
		Mastitis negative			Latent infection	Mastitis positive		
		Non-specific Cellular reaction	Teat canal infection			Septic	Aseptic	Total
			Irrelevant	Relevant				
A	Key (Table 3).....	9 (21,4%)	7 (16,7%)	17 (40,5%)	—*	8 (19,0%)	1 (2,4%)	9 (21,4%)
B	International dairy federation: Critical thresholds							
	a. 300 $\times$ 10^3 cells/ml.....	—	—	—	4 (9,5%)	28 (66,7%)	10 (23,8%)	38 (90,5%)
	b. 500 $\times$ 10^3 cells/ml.....	—	—	—	7 (16,7%)	26 (61,9%)	99 (21,4%)	35 (83,3%)

*— = diagnosis not possible by means of criteria concerned

TABLE 10 The coefficients of variation of MMT, DMC, ECC and DNA determinations on mastitis negative and positive milk samples from cows in various stages of lactation

Type of samples		Coefficient of variation (%)			
		MMT	DMC	ECC	DNA
Mastitis negative.....	Early lactation.....	12,89	86,16	109,95	34,58
	Middle lactation.....	11,98	75,69	64,20	32,17
	Late lactation.....	19,14	101,48	51,59	44,83
	Mean.....	14,67	87,78	75,25	37,19
Mastitis positive.....	Early lactation.....	11,54	84,99	66,82	31,07
	Middle lactation.....	27,72	80,39	77,04	51,08
	Late lactation.....	34,09	90,41	172,23	87,14
	Mean.....	24,45	85,26	105,36	56,43

The high reproduceability of the MMT is even more probable in view of the very low within-sample variation recorded by Mancini *et al.* (1965), i.e. 2,0% of the mean on analysis of the total variance noted in 64 observations made with a serum albuminantiserum system comparable to the MMT.

The coefficient of correlation (r) derived from the total of 126 observations made on all mastitis negative and "positive" samples in this experiment are summarized in Table 11.

A coefficient of correlation $r = +0,7$ or $r = -0,7$ indicates the existence of highly significant relationships between the variables concerned. Table 11 consequently suggests a high degree of correlation between data derived by means of MMT, DMC, ECC and DNA determinations. The statistically highly significant correlations may be due to a causal relationship between the variables concerned, but this is not necessarily so. All that is suggested by Table 11 is the existence of a straightline relationship between the variables. The following is an attempt to interpret these high correlations.

TABLE 11 The coefficients of correlation (r) between MMT, DMC, ECC and DNA determinations performed jointly on 126 mastitis negative and "positive" samples

Data from	Coefficient of correlation			
	MMT	DMC	ECC	DNA
MMT.....	1,00	0,78	0,68	0,76
DMC.....	0,78	1,00	0,74	0,66
ECC.....	0,68	0,74	1,00	0,77
DNA.....	0,76	0,66	0,77	1,00

The results obtained hitherto indicate that the MMT is the most reliable indicator of mastitis. According to Paape, Snyder & Hafs (1962), Paape, Hafs & Tucker (1963, 1964), Paape, Tucker & Hafs (1965), Hauke & Lüttigh (1966), Hauke (1967), and others reviewed by Giesecke & Van den Heever (1974), DNA determination is apparently the most accurate method for estimating the number of somatic cells present in milk. If this is correct, and the BSA content of milk rises during mastitis due to increased destruction of udder epithelium by the inflammatory process, rather than an increased permeability of the epithelial cells *per se*, one should find a low correlation between MMT and DNA in normal and high correlation in mastitic milk. A high coefficient of truly mastitis positive samples would suggest a cause-and-effect relationship between epithelial damage and increased BSA concentration and therefore provide further proof of the specificity of the MMT in the diagnosis of mastitis, i.e. inflammatory epithelial damage of the udder.

TABLE 12 The coefficients of correlation (r) between MMT, DMC, ECC and DNA determinations performed on 84 mastitis negative samples

Data from	Coefficient of correlation			
	MMT	DMC	ECC	DNA
MMT.....	1,00	0,18	0,29	0,22
DMC.....	0,18	1,00	0,44	0,30
ECC.....	0,29	0,44	1,00	0,12
DNA.....	0,22	0,30	0,12	1,00

The coefficients of correlation between the methods used for examining 84 mastitis negative samples are summarized in Table 12. All the correlations can be regarded as insignificant within a 95% confidence interval where $r = 0,1—0,4$. The data suggests that there is no significant relationship between BSA concentrations and somatic cell counts of mastitis negative milk.

TABLE 13 The coefficients of correlation (r) between MMT, DMC, ECC and DNA determinations performed on 42 "mastitis positive" samples

Data from	Coefficient of correlation			
	MMT	DMC	ECC	DNA
MMT.....	1,00	0,52	0,51	0,62
DMC.....	0,52	1,00	0,73	0,28
ECC.....	0,51	0,73	1,00	0,44
DNA.....	0,62	0,28	0,44	1,00

The coefficients of correlation between the methods applied to 42 "mastitis positive" samples are summarized in Table 13. The calculations suggest a considerable improvement in the coefficient of correlation between MMT and DNA determinations. Within a 95% confidence interval, where $r = 0,4—0,8$, the coefficient of correlation of MMT to DNA ($r = 0,62$) is significant. Whereas the relationship of MMT to DNA is considered to be a causal one the statistically highly significant coefficient of correlation of DMC to ECC ($r = 0,73$) does not characterize a causal relationship with regard to the physiological or pathological condition of the udder epithelium. It merely suggests that somatic cell counts established in "mastitis positive" samples (Table 13) by DMC and ECC are more closely related than those established by the same methods in mastitis negative samples (Table 12).

The moderately significant correlations between MMT and the cytological methods (Table 13) probably result chiefly from incorrect classification of mastitis positive samples by application of international standards.

The coefficients of correlation between the different diagnostic methods were also calculated for results obtained from 8 true cases of subclinical septic mastitis (Table 9), identified as such with the aid of MMT and the Table 3 key. The relevant figures appear in Table 14.

TABLE 14 The coeffcients of correlation (r) between MMT, DMC, ECC and DNA determinations performed on 8 true cases of subclinical septic mastitis

Data from	Coefficient of correlation			
	MMT	DMC	ECC	DNA
MMT.....	1,00	0,65	0,65	0,78
DMC.....	0,65	1,00	0,47	0,47
ECC.....	0,65	0,47	1,00	0,71
DNA.....	0,78	0,47	0,71	1,00

A highly significant causal relationship between MMT and DNA ($r = 0,78$) is clearly suggested (Table 14). The slightly lower coefficients of correlation between MMT and DMC or ECC ($r = 0,65$) suggest a less close causal relationship between BSA concentration and somatic cell counts in truly mastitic

samples and thus substantiate the diagnostic inaccuracy of the DMC and ECC. The coefficient of correlation between DMC and ECC was surprisingly low (r = 0,47), thus emphasizing a poor relationship between the two methods.

The number of truly mastitic samples examined was, however, very small, which could give rise to serious doubts regarding the validity of the results (Table 14) especially since the 95% confidence interval for MMT to DNA (r = 0,18—0,95) is indeed very wide. But a coefficient of correlation of r = 0,76 for MMT to DNA, within a 95% confidence interval of r = 0,65—0,85, was obtained earlier with negative and "positive" samples (Table 11). This suggests that examination of a large number of truly mastitic samples would probably increase, rather than decrease, the significance of the coefficient of correlation between MMT and DNA.

SERIES III: The Repeatability of MMT, DMC and ECC in Diagnosing Subclinical Mastitis in Lactating Cows

Milk collected from 74 quarters of 19 lactating cows on each of 3 successive days was examined in this investigation. The resulting data appear in Tables 15, 16 and 17.

If an MMT reading of 8 mm and somatic cell counts of 300 × 10^3 cells/ml or 500 × 10^3 cells/ml are used as thresholds to distinguish between healthy and mastitic quarters, the resulting diagnoses would be as summarized in Table 18.

According to the MMT a mean of 65,7 ± 1,5* quarters (88,7 ± 2,0%) were mastitis negative during the three consecutive examinations. Based on a critical threshold of <300 × 10^3 cells/ml, the DMC and ECC suggest that a mean of 35,3 ± 2,5 quarters (47,7 ± 3,4%) and 12,3 ± 2,5 quarters (16,7 ± 3,4%) respectively were mastitis negative. At a threshold of <500 × 10^3 cells/ml the DMC and ECC respectively show that 40,7 ± 2,1 quarters (55,0 ± 2,8%) and 23,3 ± 3,5 quarters (31,6 ± 4,7%) were mastitis negative.

With regard to the mastitis positive quarters, the MMT suggests that a mean of 8,3 ± 1,5 quarters (11,3 ± 2,0%) were mastitic over the 3 days. At a threshold of >300 × 10^3 cells/ml the corresponding values for DMC and ECC were 38,7 ± 2,5 (52,2 ± 3,4%) and 61,7 ± 2,5 (83,3 ± 3,4%) respectively; and at a threshold of >500 × 10^3 cells/ml the corresponding values for DMC and ECC were 33,3 ± 2,1 (45,0 ± 2,8%) or 50,7 ± 3,5 (68,4 ± 4,7%) respectively.

At a threshold of 300 × 10^3 cells/ml the errors for DMC and ECC were 40,9 ± 1,4% and 72,0 ± 1,4% respectively whereas they were 33,7 ± 0,08% and 57,1 ± 2,7% at 500 × 10^3 cells/ml. Thus the average error of the DMC and ECC is 37,3 ± 1,1% and 64,6 ± 2,1% respectively.

TABLE 15 The repeatability of MMT on samples drawn on 3 successive days

Day of sampling	MMT-diameters (mm)															
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	Number of quarters concerned															
Day 1.....	5	22	21	12	2	4	1	1	2	3	—	1	—	—	—	—
Day 2.....	3	15	25	13	6	2	4	2	1	1	1	—	—	—	1	—
Day 3.....	—	12	38	8	5	4	1	2	2	1	1	—	—	—	—	—

TABLE 16 The repeatability of the DMC on 3 successive days

Day of sampling	No. of samples with somatic cell counts of		
	<300 × 10^3 cells/ml	300 × 10^3—500 × 10^3 cells/ml	>500 × 10^3 cells/ml
1.....	38	5	31
2.....	35	4	35
3.....	33	7	34

TABLE 17 The repeatability of the ECC on 3 successive days

Day of sampling	No. of samples with somatic cell counts of		
	<300 × 10^3 cells/ml	300 × 10^3—500 × 10^3 cells/ml	>500 × 10^3 cells/ml
1.....	15	12	47
2.....	10	10	54
3.....	12	11	51

* Standard deviation.

TABLE 18 Mastitis diagnoses resulting from MMT, DMC and ECC performed on 3 successive days on 74 quarters of 19 lactating cows

Mastitis diagnosis	Negative										Positive									
Diagnostic threshold	MMT < 8 mm		DMC < 300 × 10^3		ECC < 300 × 10^3		DMC < 500 × 10^3		ECC < 500 × 10^3		MMT > 8 mm		DMC > 300 × 10^3		ECC > 300 × 10^3		DMC > 500 × 10^3		ECC > 500 × 10^3	
Number of quarters	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Day 1	66	89,2	38	51,4	15	20,3	43	58,1	27	36,5	8	10,8	36	48,6	59	79,7	31	41,9	47	63,5
Day 2	64	86,5	35	47,3	10	13,5	39	52,7	20	27,1	10	13,5	39	52,7	64	86,5	35	47,3	54	72,9
Day 3	67	90,5	33	44,6	12	16,2	40	54,1	23	31,1	7	9,5	41	55,4	62	83,8	34	45,9	51	68,9
$\bar{X}$	65,7	88,7	35,3	47,7	12,3	16,7	40,7	54,97	23,3	31,6	8,3	11,3	38,7	52,2	61,7	83,3	33,3	45,03	50,7	68,4
SD	1,52	2,04	2,52	3,42	2,52	3,42	2,08	2,80	3,51	4,72	1,53	2,04	2,52	3,42	2,52	3,42	2,08	2,80	3,51	4,72
CV	2,33	2,30	7,12	7,17	20,40	20,54	5,12	5,10	15,05	14,94	18,33	18,11	6,51	6,56	4,08	4,11	6,24	6,22	6,93	6,89

TABLE 19 Data obtained by application of MMT, DMC and ECC to milk from 301 quarters of 76 lactating cows under excellent management

Method	Number of quarters					
	Mastitis negative			Mastitis postive		
	Stage of lactation					
	Early	Middle	Late	Early	Middle	Late
MMT	89	165	23	9	10	5
DMC	84	149	22	14	26	6
ECC	67	117	7	31	58	21

TABLE 20a Results of bacteriological examination of teat and cisternal samples collected on 3 successive days and one day respectively from 26 lactating quarters of 7 cows

Cows and quarters	Bacteriology			
	Teat samples			Cistern samples
	Day—1	Day 0	Day+1	Day+1
RF	strept	strept	strept	0*
RH	strept	strept	strept	0
3054 LF	strept	0	strept	0
LH	strept	strept	strept	0
RF	staph	staph	staph	staph
RH	0	strept	strept	0
574 LF	strep + staph	strept + staph	strept + staph	0
LH	staph	staph	staph	0
RF	staph	staph	staph	staph
RH	0	strept	strept	0
5282 LF	staph	strept + staph	strept + staph	0
LH	staph	staph	staph	staph
RF	0	0	0	0
RH	staph	strept + staph	staph	staph
6581 LF	inactive**	inactive	inactive	inactive
LH	staph	staph	staph	staph
RF	inactive	inactive	inactive	inactive
RH	0	0	0	0
5646 LF	0	0	staph	0
LH	0	staph	0	0
RF	0	0	0	0
RH	serratia	serratia	serratia	0
8571 LF	0	0	staph	0
LH	0	0	0	0
RF	strept	strept	strept	0
RH	strept	strept	strept	0
8602 LF	staph	0	0	0
LH	0	0	0	0

*0 = no bacterial growth
Inactive** = quarters inactive

Correction of daily fluctuation (SD) in mastitis positive quarters

The daily variations in mastitis positive quarters diagnosed by the MMT resulted from fluctuations in the BSA levels not in 1,52 (Table 18) but in 3 quarters of 2 animals [Cow 3913 (RF and LH); Cow 574 (RF)]. The RF quarter of Cow 3913 was initially mastitic but apparently improved temporarily during one of the days of this investigation, while the LH quarter became mastitic. The MMT of the RF quarter of Cow 574 varied from 8 to 9 to 8 mm and *S. aureus* was recovered on all 3 days. The reliability of a diagnosis based on the MMT was therefore critically affected by 3 quarters, i.e. 4,1% and not 2,0% (Table 18), of all quarters examined. Corresponding daily variations in mastitis positive diagnoses by the DMC and ECC involved an average of 11 quarters (14,5%) of 9 cows, and 21 quarters (28,4%) of 17 cows respectively.

Such daily variations may result from genuine changes in the health of the quarters, which may become mastitic or recover from mastitis, during the period of examination. The MMT suggests that 4,1% of all quarters examined showed genuine changes in

TABLE 19 Data obtained by application of MMT, DMC and ECC to milk from 301 quarters of 76 lactating cows under excellent management

Method	Number of quarters					
	Mastitis negative			Mastitis postive		
	Stage of lactation					
	Early	Middle	Late	Early	Middle	Late
MMT	89	165	23	9	10	5
DMC	84	149	22	14	26	6
ECC	67	117	7	31	58	21

TABLE 20a Results of bacteriological examination of teat and cisternal samples collected on 3 successive days and one day respectively from 26 lactating quarters of 7 cows

Cows and quarters	Bacteriology			
	Teat samples			Cistern samples
	Day—1	Day 0	Day+1	Day+1
RF	strept	strept	strept	0*
RH	strept	strept	strept	0
3054 LF	strept	0	strept	0
LH	strept	strept	strept	0
RF	staph	staph	staph	staph
RH	0	strept	strept	0
574 LF	strep + staph	strept + staph	strept + staph	0
LH	staph	staph	staph	0
RF	staph	staph	staph	staph
RH	0	strept	strept	0
5282 LF	staph	strept + staph	strept + staph	0
LH	staph	staph	staph	staph
RF	0	0	0	0
RH	staph	strept + staph	staph	staph
6581 LF	inactive**	inactive	inactive	inactive
LH	staph	staph	staph	staph
RF	inactive	inactive	inactive	inactive
RH	0	0	0	0
5646 LF	0	0	staph	0
LH	0	staph	0	0
RF	0	0	0	0
RH	serratia	serratia	serratia	0
8571 LF	0	0	staph	0
LH	0	0	0	0
RF	strept	strept	strept	0
RH	strept	strept	strept	0
8602 LF	staph	0	0	0
LH	0	0	0	0

*0 = no bacterial growth
Inactive** = quarters inactive

Correction of daily fluctuation (SD) in mastitis positive quarters

The daily variations in mastitis positive quarters diagnosed by the MMT resulted from fluctuations in the BSA levels not in 1,52 (Table 18) but in 3 quarters of 2 animals [Cow 3913 (RF and LH); Cow 574 (RF)]. The RF quarter of Cow 3913 was initially mastitic but apparently improved temporarily during one of the days of this investigation, while the LH quarter became mastitic. The MMT of the RF quarter of Cow 574 varied from 8 to 9 to 8 mm and *S. aureus* was recovered on all 3 days. The reliability of a diagnosis based on the MMT was therefore critically affected by 3 quarters, i.e. 4,1% and not 2,0% (Table 18), of all quarters examined. Corresponding daily variations in mastitis positive diagnoses by the DMC and ECC involved an average of 11 quarters (14,5%) of 9 cows, and 21 quarters (28,4%) of 17 cows respectively.

Such daily variations may result from genuine changes in the health of the quarters, which may become mastitic or recover from mastitis, during the period of examination. The MMT suggests that 4,1% of all quarters examined showed genuine changes in

health status. Contrary to this, diagnoses based on DMC and ECC reveal critical changes in the health status of 14,5% and 28,4% of the quarters examined. In terms of the MMT therefore, 10,4% and 24,3% of the data obtained by DMC and ECC respectively showed changes in somatic cell counts which were of sufficient magnitude to simulate mastitis. Consequently it follows that although Table 18 suggests a variation of 11,3 $\pm$ 2,0% for the MMT, in terms of the corrected number of quarters shifting across the critical threshold, the daily variation of truly mastitic quarters should read 11,3 $\pm$ 4,1%, whereas the corrected average error of the DMC and ECC is 37,3 $\pm$ 10,4% and 64,6 $\pm$ 20,2% respectively. The data suggest that diagnostic accuracy and reproduceability of the DMC and ECC are poor in comparison to the MMT.

SERIES IV: Evaluation of MMT, DMC and ECC data from 76 Cows under excellent Conditions of Milking Hygiene and Management

Since the data obtained in the studies considered hitherto were derived from cows under poor management it was thought essential to conduct a comparative study on lactating cows under very good management. The results obtained by application of the MMT, DMC and ECC to samples from 301 quarters of 76 such cows are summarized in Table 19.

According to the MMT, 24 (7,9%) of the quarters were mastitic, i.e. 2,9%; 3,3% and 1,7% were mastitic in early, middle or late lactation respectively.

According to the DMC 46 quarters (15,3%) were mastitic when a diagnostic threshold of $<500 \times 10^3$ cells/ml was used, i.e. 4,7%; 8,7% and 1,9% of the quarters of cows in early, middle and late lactation respectively. With the ECC the corresponding figures were a total of 110 quarters (36,9%), i.e. 10,3%; 19,3% and 7,3% of the quarters of cows in early, middle or late lactation.

In terms of the MMT results, 7,4% and 29,0% of quarters were incorrectly classified as mastitis positive by the DMC and ECC respectively.

Results obtained by very sensitive counting methods such as the ECC are more likely to be affected by cytological changes in milk elicited by a wide variety of factors than is the case with less sensitive methods such as the DMC. Thus Table 19 suggests that the ECC is more readily rendered inaccurate by involutionary changes of the mammary gland than the DMC or MMT.

SERIES V: The Effect of Teat Canal Infections on the Diagnosis of Mastitis

The existence of teat canal infections can be demonstrated by examination of samples collected aseptically in parallel via the teat canal and from the teat cistern by puncture of the teat wall. If bacteria are found only in the former a teat canal infection is present. Whereas bacteriological data obtained from such samples can be regarded as reliable, this does not apply to cytological and immunochemical data from the cisternal samples due to frequent admixture of blood. The results of bacteriological, cytological and immunochemical examinations on 26 lactating quarters of 7 cows sampled accordingly are summarized in Tables 20a and 20b.

TABLE 20b Results of cytological and immunochemical examination of teat samples collected on 3 successive days from 26 lactating quarters of 7 cows

Cows and quarters	Somatic cell counts ($\times 10^3$/ml)			MMT diameters (mm)		
	Day -1	Day 0	Day $+1$	Day -1	Day 0	Day $+1$
RF	2 500	1 150	2 400	5	5	4
RH	3 150	1 000	1 150	5	5	5
3054 LF	2 050	3 950	1 000	5	5	4
LH	150	750	350	5	4	4
RF	4 950	3 750	7 500	10	10	9
RH	150	50	50	5	4	4
574 LF	1 500	1 150	1 100	7	6	5
LH	600	200	400	5	5	5
RF	8 550	9 200	5 750	11	12	10
RH	1 550	500	850	5	5	4
5282 LF	1 750	2 350	2 500	6	9	7
LH	7 000	7 200	7 650	9	9	8
RF	50	50	150	6	5	5
RH	300	1 150	2 000	6	5	8
6581 LF	inactive*	inactive	inactive	inactive	inactive	inactive
LH	350	1 000	650	9	5	10
RF	inactive	inactive	inactive	inactive	inactive	inactive
RH	1 000	4 400	1 330	5	5	4
5646 LF	1 700	450	3 500	4	5	4
LH	2 800	2 950	1 750	4	5	4
RF	50	50	100	5	4	4
RH	9 150	5 200	3 500	9	6	5
8571 LF	50	50	150	5	4	5
LH	100	50	150	4	4	4
RF	250	200	350	3	4	4
RH	200	800	400	3	3	4
8602 LF	50	150	200	4	4	4
LH	50	100	50	4	4	4

Inactive* = quarters inactive

TABLE 21a Diagnoses obtained by international standard, MMT and a combination of the two from teat samples of 26 lactating quarters of 7 cows on 3 successive days

Cow and quarters		Diagnoses								
		International standard*			MMT			MMT + International standard		
		Day — 1	Day 0	Day + 1	Day — 1	Day 0	Day + 1	Day — 1	Day 0	Day + 1
3054	RF	ssm*	ssm	ssm	n	n	n	rti	rti	rti
	RH	ssm	ssm	ssm	n	n	n	rti	rti	rti
	LF	ssm	am	ssm	n	n	n	rti	ncr	rti
	LH	li	ssm	li	n	n	n	iti	rti	iti
574	RF	ssm	ssm	ssm	p	p	p	ssm	ssm	ssm
	RH	n	li	li	n	n	n	n	iti	iti
	LF	ssm	ssm	ssm	n	n	n	rti	rti	rti
	LH	ssm	li	li	n	n	n	rti	iti	iti
5282	RF	ssm	ssm	ssm	p	p	p	ssm	ssm	ssm
	RH	am	ssm	ssm	n	n	n	ncr	rti	rti
	LF	ssm	ssm	ssm	n	p	n	rti	ssm	rti
	LH	ssm	ssm	ssm	p	p	p	ssm	ssm	ssm
6581	RF	n	n	n	n	n	n	n	n	n
	RH	li	ssm	ssm	n	n	p	iti	rti	ssm
	LF	inactive**	inactive	inactive	inactive	inactive	inactive	inactive	inactive	inactive
	LH	li	ssm	ssm	p	n	p	ssm	rti	ssm
5646	RF	inactive	inactive	inactive	inactive	inactive	inactive	inactive	inactive	inactive
	RH	am	am	am	n	n	n	ncr	ncr	ncr
	LF	am	n	ssm	n	n	n	ncr	n	rti
	LH	am	ssm	am	n	n	n	ncr	rti	ncr
8571	RF	n	n	n	n	n	n	n	n	n
	RH	ssm	ssm	ssm	p	n	n	ssm	rti	rti
	LF	n	n	li	n	n	n	n	n	iti
	LH	n	n	n	n	n	n	n	n	n
8602	RF	li	li	li	n	n	n	iti	iti	iti
	RH	li	ssm	li	n	n	n	iti	rti	iti
	LF	li	n	n	n	n	n	iti	n	n
	LH	n	n	n	n	n	n	n	n	n

* Threshold = 500 × 10^3 cells/ml: am = aseptic mastitis; iti = irrelevant teat canal infection; li = latent infection; n = mastitis negative; ncr = nonspecific cellular reaction; p = mastitis positive; rti = relevant teat canal infection; ssm = septic subclinical mastitis

inactive** = quarters inactive

TABLE 21b Summary of diagnoses established by MMT, international standard and MMT plus international standard on teat samples from 26 lactating quarters of 7 cows

Diagnostic standards	Days of examination	Diagnosis							
		Mastitis				Latent infection	Non-specific cellular reaction	Teat canal infection	
		Negative	Positive					Relevant	Irrelevant
			Total	Aseptic	Septic				
International.....	Day—1....	6	14	4	10	6	—*	—	—
	Day 0.....	7	16	2	14	3	—	—	—
	Day+1....	5	15	2	13	6	—	—	—
MMT.....	Day—1....	21	5	—	—	—	—	—	—
	Day 0.....	22	4	—	—	—	—	—	—
	Day+1....	21	5	—	—	—	—	—	—
MMT+International	Day—1....	21	5	0	5	—	4	6	5
	Day 0.....	22	4	0	4	—	2	10	3
	Day+1....	21	5	0	5	—	2	8	6

—* =Diagnosis not possible by means of criteria concerned

Teat canal samples collected on 3 successive days were analyzed by application of 3 diagnostic methods, namely the international standard, the MMT alone and the key in Table 3 (Table 21a and 21b).

Subclinical mastitis was diagnosed in 57,7 ± 3,9% (15 ± 1) and 17,9 ± 2,2% (4,7 ± 0,6) quarters respectively by application of the international standard and the MMT. Since the bacteriological results from cistern samples collected on the 3rd day (Table 20a) agreed with those of the MMT (Table 21a), it could be calculated that 39,7 ± 1,7% of positive mastitis diagnoses established by international standards were incorrect.

Examination of cistern samples confirmed the reliability of the diagnostic key suggested (Table 3). According to the international standard there were several cases of aseptic mastitis (8) and latent infection (15) (Table 21b). However, the MMT revealed that the 8 cases of aseptic mastitis had non-specific cellular reactions, not related to udder inflammation, and bacteriological examination of cistern samples did not detect any evidence of latent udder infections. Moreover, use of the key revealed that the majority of quarters (24 out of 37), diagnosed as having subclinical septic mastitis by international standards, were in fact not mastitic but had relevant teat canal infections.

It might be argued that the distinction between mastitis and relevant teat canal infections is of academic rather than of practical importance because such teat canal infections eventually result in mastitis and have to be treated as such. Although this argument appears to be valid in principle, it is at present scientifically unsound. It has been shown above that teat canal infections are not synonymous with mastitis. The widely used conventional diagnostic methods, on which most of our information concerning the aetiology, pathogenesis, incidence, chemotherapy and prophylaxis of mastitis is based, cannot distinguish between teat canal infections and true mastitis.

This has rather disconcerting implications regarding the accuracy of our knowledge of the disease. Forbes (1970a), for example, monitored 72 quarters by monthly cisternal puncture and found only one that remained completely uninfected throughout lactation. However, with the exception of the clinical cases of mastitis observed, he failed to demonstrate conclusively that the bacteria isolated were in fact associated with mastitis and had not grown when the phagocytes which had devoured them in the proximal portions of the teat canal deteriorated on culturing before digesting them. More detailed data concerning the frequency with which true mastitis results from teat canal infections, diagnosed according to Table 3, were recently obtained by Giesecke & Du Toit (1973, unpublished data). These results were obtained from 629 quarters of 158 lactating cows examined twice within a period of six weeks. They suggest that, in contrast to common belief, the lactating bovine mammary gland has a very efficient defense mechanism. From a total of 212 quarters with potentially mastitogenic teat canal infections caused by *S. aureus* (67 quarters) or *S epidermidis* (145 quarters), only 22 (19 × *S. aureus*, 3 × *S. epidermidis*) developed subclinical septic mastitis. In 65 quarters (19 × *S. aureus*; 46 × *S. epidermidis*) the infection remained confined to the teat canal and the micro-organisms were completely eliminated from 125 teat canals (34 × *S. aureus*; 91 × *S. epidermidis*).

"Udder" infection by micro-organisms other than *Str. agalactiae*, especially *S. aureus*, are being reported with increasing frequency and represent an acknowledged major problem in bovine mastitis (Wilson, 1963; Schalm & Lasmanis, 1957; Renk, 1961; Rendel & Sundberg, 1962; Heidrich & Renk, 1963; Giesecke, Van den Heever & Du Toit, 1972). *S. aureus* infections are known to be associated with teat canal lesions resulting from mechanical milking (McDonald, 1970) which is accepted as having a deleterious effect on bovine udder health (Happel, 1963a, b; Fell, 1964; Walser, 1966a, b; Brodauf, 1968; Trautwein, Englert & Brodauf, 1958). The relationships between mechanical milking and teat lesions or resulting infections by *S. aureus* respectively have not been assessed. However, disregard of such teat canal infections may well be a reason for the present highly unsatisfactory mastitis situation (Morse, 1970). It probably also explains why Hilpert & Enkelmann (1964) in an earlier assessment concluded that a radial immunodiffusion test for BSA was not a reliable method for the diagnosis of mastitis.

TABLE 22 The effect of administration into the *ductus papillaris* of staphylococcal *beta* toxin in polyacrylamide gell on mastitis diagnosis

Cows and quarters		Treatments on Day 0	Results									
			Bacteriology				Somatic cell counts ($\times 10^3$ cells/ml)			MMT diameters (mm)		
			Teat samples			Cistern samples						
			Day — 1	Day 0	Day + 1	Day + 1	Day — 1	Day 0	Day + 1	Day — 1	Day 0	Day + 1
	RF	P* + T**	0†	0	0	0	50	150	10 350	4	4	10
	RH	P + T	0	strept	0	0	50	50	8 500	3	4	8
7662	LF	—	0	0	0	0	250	100	350	4	4	4
	LH	—	0	0	0	0	100	50	150	4	4	4
	RF	P + T	0	staph	0	0	50	50	UC††	4	4	10
	RH	P + T	0	*E. coli*	0	0	50	50	UC	4	5	9
7644	LF	—	*Sarcina sp.*	strept	staph	0	50	1 300	350	5	5	5
	LH	—	staph	strept	strept	0	50	50	200	5	4	5
	RF	P + T	0	0	0	0	50	50	UC	3	3	11
	RH	P + T	0	0	0	0	50	50	4 750	3	3	7
7028	LF	—	0	0	0	0	50	50	100	3	4	4
	LH	—	0	0	0	0	50	50	150	3	4	4
	RF	P + T	0	staph	0	0	50	50	6 350	4	4	11
	RH	P + T	0	0	0	0	50	50	8 750	3	5	9
6860	LF	—	0	staph	0	0	50	50	50	3	5	5
	LH	—	0	0	0	0	50	50	50	4	5	4
	RF	P + T	0	strept	0	0	50	50	UC	3	5	12
	RH	P + T	0	strept	0	0	50	50	5 500	3	5	14
9303	LF	—	0	0	0	0	50	50	50	3	4	4
	LH	—	0	strept	0	0	50	50	50	3	4	4
	RF	—	0	0	0	0	50	50	50	4	4	4
	RH	I***	0	0	0	0	50	50	50	3	3	4
9306	LF	P	0	0	0	0	50	50	550	4	3	4
	LH	—	0	0	0	0	50	50	50	4	4	4
	RF	—	0	0	0	0	150	100	150	4	4	4
	RH	I	0	0	0	0	50	50	100	4	4	4
8562	LF	P	0	0	0	0	100	100	650	4	4	4
	LH	—	0	0	strept	0	100	150	250	4	4	4

*P = polyacrylamide gel; **T = staphylococcal *beta* toxin; ***I = brief intraductular insertion of nozzle
†O = bacteriologically negative; ††UC = innumerable number of somatic cells

SERIES VI: The Effect of Staphylococcal beta Toxin Administered into the Teat Canal on the Diagnosis of Mastitis

Although no bacteria could be found in cisternal samples collected from quarters with teat canal infections in the foregoing experiment, somatic cell counts were increased. This suggested that cellular reactions might be elicited by bacterial toxins diffusing proximally through the teat canal rather than by viable bacteria entering the teat cistern.

In an attempt to simulate a slow release of bacterial toxin in the teat canal, sterile staphylococcal *beta* toxin was suspended in polyacrylamide gel and administered into the right 10 teat canals of 5 cows. The results obtained are summarized in Table 22.

Comparison of data obtained from samples collected 24 hours after toxin administration (Day + 1) with those taken prior to this (Day − 1; Day 0) shows pronounced cellular reactions in all treated right quarters, whereas no such response occurred in the untreated left quarters. Since all cistern samples collected 24 hours after treatment were bacteriologically negative, the cellular reactions were due to the administered toxin plus polyacrylamide gel. Polyacrylamide gel, administered alone, only elicited a much slighter, but significant cellular reaction. The MMT shows that 0,06 ml of staphylococcal *beta* toxin, and not the polyacrylamide gel was sufficiently potent to initiate aseptic mastitis after only 5 hours in the teat canal.

Since it appears unlikely that such large quantities of toxin would be synthesized *in vivo* by micro-organisms inhabiting exclusively the teat canal, the aseptic mastitis elicited is considered to be an extreme type of reaction rather than that which would occur under natural conditions. Differences in the BSA levels, reflected by MMT diameters that ranged from 7 to 14 mm 24 hours after administration of toxin, suggest that the toxin causes variable increases of BSA levels in milk, which depend on the quantity of the toxin affecting the udder epithelium over a given period of time after diffusing into the cisternal lumen. The BSA content, in turn, depends upon the degree of epithelial damage, i.e. a gradient of BSA concentrations from normal to very high can be expected to occur. The possibility that irritants administered intracisternally would cause such rapid and severe epithelial damage that the BSA concentrations in milk of such quarters would eventually approach an all-or-none phenomenon as found by Lecce & Legates (1959) is, however, not precluded.

This experiment (Table 22) and the previous one (Tables 20a, b) show clearly that cellular reactions can be elicited by teat canal infections in the absence of raised BSA levels of inflammatory magnitude. Schalm *et al.* (1971) found that increased BSA levels precede leucocytic migration by 2 to 3 hours initially and the latter also lags behind the former at the termination of udder inflammation induced by intracisternal administration of appropriate irritants. Thus cellular reactions associated with teat canal infections without concurrently increased BSA concentrations could result from terminating inflammatory reactions in the quarters concerned. On the other hand Schalm *et al.* (1971) also found that "the length of time between introduction (intracisternal) of the irritant, the appearance of BSA, and the magnitude of BSA concentrations are conditioned by the severity of the shock imposed on the gland". Consequently the cellular reactions associated with the teat canal infections could also result from stimuli, such as very small amounts of bacterial toxin or other metabolites, capable of causing leucocytic reaction but incapable of eliciting epithelial damage and increased BSA concentrations. This represents a pre-inflammatory leucocytosis discussed elsewhere (Giesecke & Du Toit, 1974; unpublished data).

Conclusions

This investigation confirms previous findings that normal milk contains a low concentration of BSA. It remains low until the 6th month (Rolleri *et al.*, 1955; Jenness *et al.*, 1956) or, as suggested by this study the 9th to 10th month of lactation. In normally lactating udders any elevation above a specific level from about the 7th day after calving until the 9th to 10th month of a lactation period indicates an inflammatory reaction of the udder epithelium viz. mastitis. Contrary to demonstration of the specificity of the BSA level for the detection of inflammation and irritation of the udder tissue, this investigation has substantiated and extended previous observations that the presence of high somatic cell counts and bacteria do not necessarily indicate inflammation of the mammary gland.

Data from this investigation clearly show that the major proportion of bacteriologically positive results, obtained by examining conventionally collected milk samples, are due to teat canal infections. Such infections frequently coincide with increased somatic cell counts in milk. However, since elevated cell counts may be due to a wide range of stimuli (Giesecke & Van den Heever, 1974), including the release of bacterial toxin in the teat canal, it is apparent that the combined presence of bacteria and many cells is not diagnostic for subclinical mastitis in lactating cows, as implied by the international definition of mastitis (Kästli, 1967; Tolle, 1971). Statistical analysis of data provided by this investigation suggests that 43,13 ± 20,87% of positive mastitis diagnoses based on the international standard may be incorrect. Although this figure is presented as a guide only, because considerable variations may be obtained when dairy herds under different systems of management are examined, it clearly emphasizes the importance of mastitic simulations by teat canal infections.

The primary objective of any method employed for the diagnosis of mastitis must be the accurate detection of inflammation. The MMT is capable of doing this with great accuracy and reproduceability. From the data obtained from 1 008 quarter samples from 179 dairy cows it seems justifiable to conclude that the MMT is the most accurate method available at present for the diagnosis of subclinical mastitis in lactating cows, especially if it is combined with bacteriological examination and somatic cell counts. Under the conditions existing in this experiment a threshold MMT value of 8 mm is suggested as demarcating mastitis negative and mastitis positive lactating cows. It must, however, be stressed that antiserum to BSA will have to be standardized if this threshold is to be used by other laboratories. Application of this standard revealed that some 4,7% of quarters were doubtful cases of mastitis because MMT reactions ranged close to 8 mm. This figure could be reduced to 2,9% by concurrent consideration of positive bacteriological cultures and high cell counts. The remaining 2,9% of quarters showed increased BSA concentrations, low cell counts and the absence of bacterial growth and could not be classified as either negative or positive for mastitis.

The superior diagnostic accuracy of the MMT is further emphasized by its high degree of repeatability, clearly illustrated by a coefficient of variation of 14,67% as opposed to 87,78%; 72,25% and 37,19% for the DMC, ECC and DNA determinations respectively.

Due to considerable variation in the somatic cell count of normal milk the coefficient of correlation between MMT, DMC, ECC and DNA determinations was found to be statistically insignificant. The coefficient of correlation between these methods improves in milk samples classified as "mastitis positive" according to international standards. It becomes highly significant between MMT and DNA if data from truly mastitic, lactating udders only are taken into account, suggesting a distinct cause-and-effect relationship between epithelial damage and elevated BSA levels.

Since the MMT is a reliable method for the diagnosis of mastitis in lactating cows, and because the MMT in combination with conventional cytological and bacteriological methods facilitates the diagnosis of teat canal infections without resorting to puncture of the gland cistern, the diagnostic potential of the MMT is considerable.

This easily performed test can be used to great advantage by mastitis control organizations or by individual practitioners for the specific diagnosis of subclinical mastitis in lactating cows. BSA is a whey protein (Larson & Kendall, 1957), hence it is unaffected by souring of milk samples. Because the MMT facilitates the identification of teat canal infections which, with regard to *S. aureus* infections, are an indication of teat canal lesions (McDonald, 1970), the test can also be employed for the detection of teat canal erosions in dairy herds.

As far as research on the chemotherapy of mastitis is concerned, the MMT could serve as an objective and accurate measure of udder irritation resulting from the intramammary administration of drugs. It is a valuable tool to use in studies on the aetiology and pathogenesis of bovine mastitis, regardless of whether the latter is due to udder infections or to mechanical or chemical trauma of the udder epithelium.

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