

THE ISOLATION AND IDENTIFICATION OF AN ANTIGEN FOR THE DIAGNOSIS OF BOVINE MASTITIS BY RADIAL IMMUNODIFFUSION

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ABSTRACT

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An antigen associated with the somatic cells obtained from mastitic milk, which is also present in the whey and can be used for the diagnosis of mastitis in a radial immunodiffusion (RID) test, has been purified and identified. It is water soluble, with an approximate pI of 4.4 and $S_{20w}=4.35$ S. The antigen was shown to be immunologically identical to bovine serum albumin by means of Ouchterlony and immunodiffusion techniques. The use of monospecific rabbit antiserum facilitates standardization of the test results.

INTRODUCTION

Mastitis is one of the most costly diseases confronting the dairy farmer (Janzen, 1970) and reliable diagnostic methods are required especially for the detection of subclinical udder inflammation which is difficult to diagnose (Giesecke & Van den Heever, 1974) by criteria currently available (Kästli, 1967; Tolle, 1971).

The diagnosis of subclinical mastitis is based mainly on bacteriological examinations in conjunction with quantitative determinations of somatic cells in milk (Tolle, 1971). For the latter purpose, the California Mastitis Test of Schalm & Norlander (1957), the direct microscopic cell count of Prescott & Breed (1910) and the electronic cell count (Tolle, Zeidler & Hesch, 1966), are probably most widely employed (Heidrich & Renk, 1967; Schalm, Carroll & Jain, 1971).

However, neither the presence of pathogenic bacteria in aseptically collected teat samples (Giesecke, Van den Heever, Hope & Van Staden, 1968) nor an increased somatic cell count necessarily indicates subclinical mastitis (Cullen, 1966; Schalm & Lasmanis, 1968; Giesecke & Van den Heever, 1974). Therefore tests based on factors such as milk enzyme activity and protein concentration have also been suggested (Peskest, 1932; Hilpert & Enkelman, 1964).

Morris & Hobbs (1971), using a polyvalent antiserum to the cells present in mastitic milk, described an RID test for the diagnosis of mastitis and observed a good correlation between the size of the largest and most distinct precipitation ring formed and the cell count. The heterogeneity of the antigen, however, resulted in the formation of a number of precipitation rings. Giesecke, Van den Heever, Du Toit & Beyer (1973) suggested that purification of the antigen which is responsible for the development of the main precipitation ring and the preparation of a specific antiserum to the antigen should improve the specificity and facilitate standardization of the test.

This communication reports the purification and identification of the antigen from mastitic milk.

MATERIALS AND METHODS

Antigens

The two major antigens A and B used during this investigation were obtained from aliquots of a milk sample from a clinical case of mastitis.

Antigen A consisted of washed milk sediment processed according to Morris & Hobbs (1971), while antigen B was a whey-protein isolated and identified as follows:

Isolation of various protein fractions from whey

Casein was precipitated from the milk at room temperature by lowering the pH to 4.5–4.6 by the gradual addition of 1.0 M HCl. The precipitate was removed by centrifugation at 1 500 g for 10 min and the supernatant whey dialyzed for 16 h in distilled water at 4 °C.

The pH of the dialyzed whey was adjusted to 8.0 with NaHCO₃ and protein precipitated by Rivanol* added to a final concentration of 10 Mm. The precipitate was recovered by centrifugation at 10 000 g for 10 min and redissolved in Tris/glycine buffer at pH 8.2. Final purification was by zone electrophoresis of the protein solution in a sucrose gradient, described by MacLeod (1967), using the same Tris/glycine buffer. Protein fractions were collected and monitored by means of a Uvicord†.

Demonstration and further purification of Antigen B

An antiserum was produced against each of the protein fractions thus separated. For this purpose equal volumes of a 0.5% (m/v) solution of the fraction concerned were emulsified with Freund's complete adjuvant. Two injections of 1.0 ml of the mixture were given subcutaneously to rabbits at an interval of 1 week. The rabbits were bled a week after the 2nd injection; the serum was separated and stored at –20 °C.

RID plates and petri dishes were produced as described by Mancini, Vaerman, Carbonara & Heremans (1963). The appropriate rabbit antiserum was diluted tenfold in 1% agarose and the mixture was then poured onto glass slides or into petri dishes to a thickness of 2 mm. The antigen was placed in circular wells, 2mm in diameter, punched into the gel 1.5 cm apart. The plates or petri dishes were left at room temperature and the size of the precipitin rings measured with the aid of callipers after 16 and 48 hours.

The partially purified antigen, which produced a precipitation ring with the closest resemblance to the largest distinct precipitation ring obtained with Antigen A, was designated Antigen B. It was subjected to further purification by zone electrophoresis and a specific antiserum to the purified antigen was produced as mentioned above.

Identification of Antigen B

Immunoabsorption (Tozer, 1967) was used to identify the partially purified and purified Antigen B and the fraction of Antigen A which was responsible for the

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largest distinct outer precipitation zone. For this purpose 1 ml of an antiserum to somatic cells in mastitic milk, prepared in rabbits as described above, was added to 1 ml of a 0,5% (m/v) solution of partially purified or purified Antigen B. After centrifugation to remove the precipitate, the supernatant was used as antiserum in an RID test with unadsorbed antiserum as control.

Polyacrylamide gel electrophoresis (PAGE) served to confirm the homogeneity of the purified Antigen B. The apparatus* used allows the tubes to be completely immersed in the anode buffer solution for more effective cooling. The discontinuous Tris/glycine buffer system of Ornstein (1964) and Davis (1964) was used with a 10% gel concentration.

Isoelectric focussing of the purified Antigen B was performed by the polyacrylamide gel method described by Catsimpoilas (1969). A duplicate gel was cut into 1 cm sections and each portion homogenized with boiled distilled water for pI determination.

The *sedimentation constant* of a 0,2% solution of the purified Antigen B in water at 20 °C was determined, using a Spinco Model E ultracentrifuge.

Cellulose acetate electrophoresis (CAE) was employed to compare the electrophoretic mobility of the purified Antigen B and bovine serum albumin (BSA), using a Microzone electrophoresis cell† with 0,075 M sodium diethylbarbiturate/HCl at pH 8,6 as buffer according to Kohn (1968).

Ouchterlony and *electroimmunodiffusion* techniques (Crowle, 1961) were used on 0,1% (m/v) solutions of purified Antigen B and BSA to confirm that they were identical.

RESULTS

Observations with antiserum to Antigen A

Antiserum from rabbits immunized with washed somatic cells from mastitic milk (Antigen A) produced multiple rings on RID. There were 3 principal precipitation rings in whole mastitic milk (Fig. 1). However, depending on the particular sample, the definition of the outer ring varied from distinct (Fig. 1) to faint (Fig. 2a). Since this variation occurred frequently the antigenic fraction responsible for the second largest precipitation ring, which was a constant feature, was selected for identification.

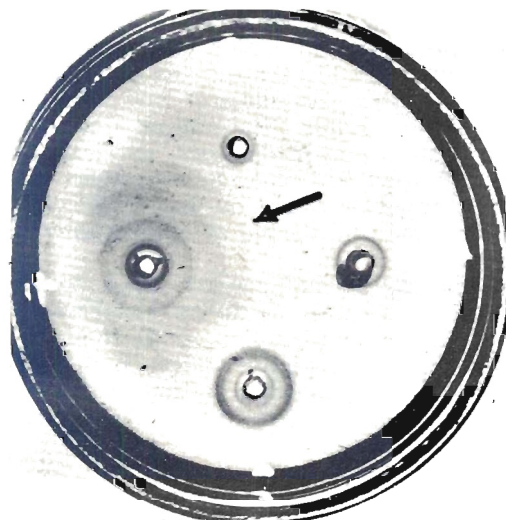


FIG. 1 In contrast to mastitis negative milk (wells without arrows), whole mastitic milk produced 3 principal precipitation rings (arrow) on RID with antiserum to Antigen A

* Own design

† Beckman Instruments

Observations with antiserum to Antigen B

One of the partially purified fractions obtained by zone electrophoresis (Antigen B) produced a distinct large outer and a faint inner ring in an RID plate (Fig. 2b). This fraction adsorbed the antibodies to the antigenic fraction of Antigen A selected for identification (Fig. 3a, b), thereby confirming that partially purified Antigen B contained the relevant antigen.

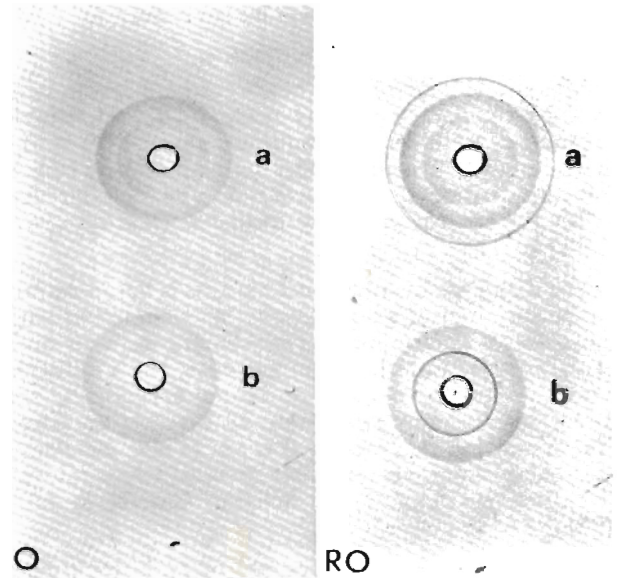


FIG. 2 Antiserum to Antigen A frequently produced a very faint extreme outer precipitation ring with other samples of mastitic milk, whereas the second largest ring remained well defined (a). A precipitation ring comparable to the latter was obtained with partially purified Antigen B (b); (O=original; RO=retouched original)

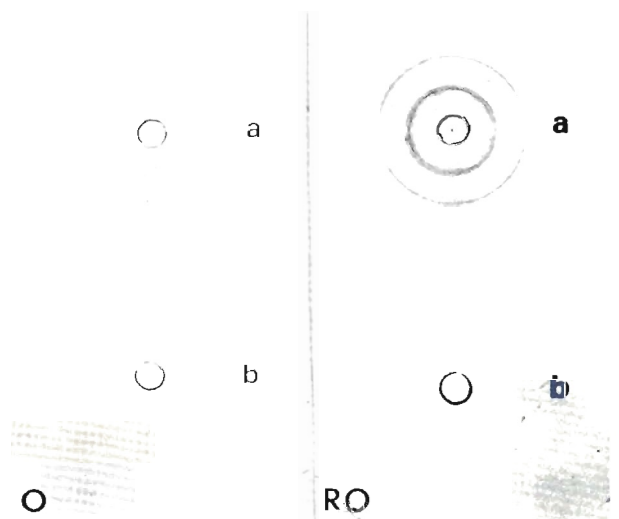


FIG. 3 After adsorption with partially purified Antigen B, antiserum to Antigen A failed to precipitate the antigen selected for identification from either mastitic milk (a) or partially purified Antigen B (b); (O=original; RO=retouched original)

Identification of purified Antigen B

The purified Antigen B produced a single band on PAGE, thereby verifying the purity of the compound under investigation. Isoelectric focussing indicated that the antigen had a pI of approximately 4,4. Ultra centrifugation showed a single peak of 4,35 S. This information, together with its solubility in water, suggested that the antigen in question was probably BSA.

Purified Antigen B and BSA had identical electrophoretic mobilities on CAE (Fig. 4) and also cross-reacted antigenically when subjected to Ouchterlony (Fig. 5) and electroimmunodiffusion tests (Fig. 6). It was therefore concluded that the antigen is BSA.

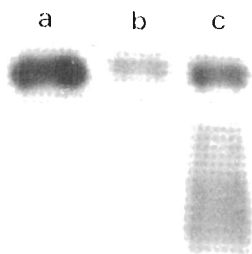


FIG. 4 The mobility of BSA (a), purified Antigen B (b) and albumin in bovine serum (c) was identical in cellulose acetate electrophoresis



FIG. 5 Complete cross reaction between bovine serum albumin (a) and purified Antigen B (b) on Ouchterlony gel diffusion. To compensate for the high motility of serum albumin, antiserum to Antigen B was applied to the unidentified well 16 h before the antigens

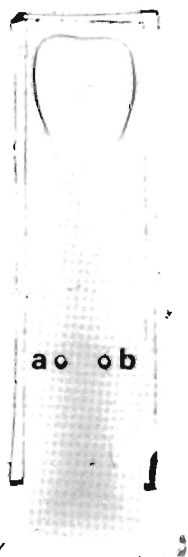


FIG. 6 Immunoelectrophoresis of BSA (a) and purified Antigen B (b) produced identical precipitation arcs. The trough contained antiserum to Antigen B

RID with the purified Antigen B

A single precipitation ring was obtained when rabbit antiserum prepared against the purified Antigen B was used in an RID test (Fig. 7) which also demonstrated the effect of antigen concentration on the size of the precipitation ring. These results are plotted graphically in Fig. 8. Precipitation rings produced by antigen concentrations of $\leq 1,5$ mg/ml reached their maximum diameter after 16 h, and a linear relationship was observed to exist between their square values and corresponding concentrations of antigen.

The diameters of precipitation rings produced by higher concentrations of antigen increased with time (Fig. 8), but, as shown by Mancini *et al.* (1963) eventually with progress of time, also give a straight line relationship.

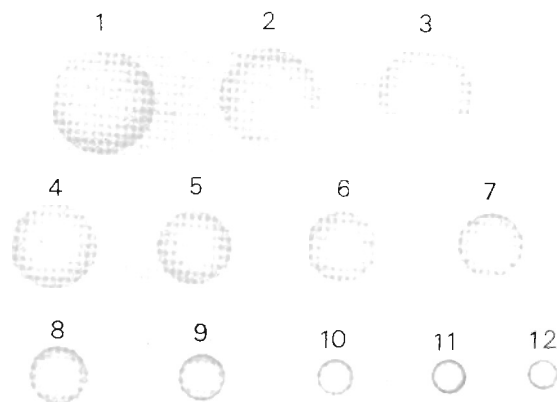


FIG. 7 A single precipitation ring was obtained with purified Antigen B against specific antiserum in an RID test. Ring size is proportional to the antigen concentration which was 20; 15; 10; 5; 2,5; 2,0; 1,5; 1,0; 0,5; 0,25; 0,20 and 0,10 mg/ml in the wells 1 to 12 respectively

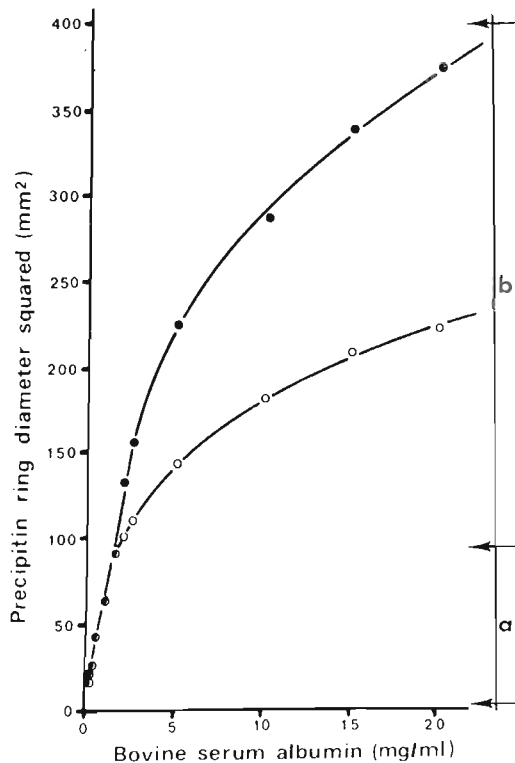


FIG. 8 The relationship between BSA concentrations and precipitation diameters squared after 16 h (o—o) and 48 h (●—●) is linear (section a) for BSA $\leq 1,5$ mg/ml and progressively linear (section b) for BSA $\geq 2,0$ mg/ml.

DISCUSSION

This investigation has shown that BSA is one of the antigens associated with the somatic cells from mastitis milk used in the test developed by Morris & Hobbs (1971). It is known that this antigen is also present in milk as such. Peskett (1932) suggested that serum albumin might increase during the secretion of abnormal milk, and a component of milk whey was found to be identical with BSA (Coulson & Stevens, 1950; Polis, Shmukler & Custer, 1950).

In contrast, Morris & Hobbs (1971) suggested that their cell-associated major antigen was immunoglobulin. However, they did not compare their antigen with a known immunoglobulin by means of immunoadsorption, immunoelectrophoresis or Ouchterlony tests. Therefore proof of the nature of their antigen was not presented.

Various studies have been done on fluctuations in the concentration of protein in abnormal milk (Carroll, 1961; Bortree, Carroll & Schalm, 1962; Shah & Morse, 1964). After the onset of experimentally induced mastitis there is an increase in the concentration of BSA and immunoglobulins, with a decrease in β -lactoglobulin and α -lactalbumin. Hilpert & Enkelmann (1964) examined milk by RID using antisera to BSA, bovine immunoglobulin and bovine β -lipoprotein. Their results also showed increased levels of BSA in mastitic milk.

BSA concentrations in normal milk are elevated at commencement (range 0,1–0,4 mg/ml) and termination (range 0,3–2,9 mg/ml) of a lactation period and seem to average 0,15 mg/ml (range 0,1–0,2 mg/ml) during mid-lactation (Dixon, Weigle & Vazquez, 1961). Preliminary tests (Viljoen, unpublished data, 1973) indicate that BSA concentrations of mastitis negative foremilk and strippings give precipitation zones with diameters (means \pm standard deviation) of $3,43 \pm 0,97$ mm and $4,25 \pm 0,98$ mm respectively. Mature milk containing more than 0,5 mg BSA/ml can be regarded as suspect for mastitis. From Fig. 8 it can be seen that the precipitation ring of antigen solutions $\leq 1,5$ mg/ml reached their maximum size in 16 hours. Overnight diffusion at room temperature (about 20–24 °C) should therefore be long enough to detect all abnormal levels of BSA in mastitic milk.

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REFERENCES

- BORTREE, A. L., CARROLL, E. J. & SCHALM, O. W., 1962. Whey protein patterns of milk from cows with experimentally produced mastitis. *J. Dairy Sci.*, 45, 1465–1471.
- CARROLL, E. J., 1961. Whey proteins of drying-off secretions, mastitic milk and colostrum separated by ion-exchange cellulose. *J. Dairy Sci.*, 44, 2194–2211.
- CATSIMPOOLAS, N., 1969. Disc Immunoelectrofocussing: Application to rabbit serum proteins. *Immunochem.*, 6, 501–502.
- COULSON, E. J. & STEVENS, H., 1950. The serological relationship of bovine whey albumin to serum albumin. *J. Biochem.*, 187, 355–363.
- CROWLE, A. J., 1961. Immunodiffusion. New York-London: Academic Press.
- CULLEN, G. A., 1966. Cells in milk. *Vet. Bull.*, 36, 337–346.
- DAVIS, B. J., 1964. Disc Electrophoresis—II: Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.*, 121, 404–427.
- DIXON, F. J., WEIGLE, W. O. & VAZQUEZ, J. J., 1961. Metabolism and mammary secretion of serum proteins in the cow. *Lab. Invest.*, 10, 216–237.
- GIESECKE, W. H. & VAN DEN HEEVER, L. W., 1974. The diagnosis of bovine mastitis with particular reference to subclinical mastitis: A critical review of relevant literature. *Onderstepoort J. vet. Res.* (In press.)
- GIESECKE, W. H., VAN DEN HEEVER, L. W., DU TOIT, I. J. & BEYER, M. C. E., 1973. The diagnosis of bovine mastitis: A critical evaluation of a polyvalent radial immunodiffusion test and other methods. *Onderstepoort J. vet. Res.*, 40, 57–66.
- GIESECKE, W. H., VAN DEN HEEVER, L. W., HOPE, HOPE, D. C. & VAN STADEN, J. J., 1968. Laboratory mastitis diagnosis: The microbiological content of parallel teat and gland cistern milk samples from quarters of known status. *Jl S. Afr. vet. med. Ass.*, 39, 33–44.
- HEIDRICH, H. J. & RENK, W., 1967. Diseases of the mammary glands of domestic animals. Philadelphia & London: Saunders.
- HILPERT, H. & ENKELMAN, D., 1964. Ein einfacher immunologischer Test zur Erkennung von Milch aus Milchdrüsen mit anomaler Sekretion. *Milchwissenschaft*, 19, 475–480.
- JANZEN, J. J., 1970. Economic losses resulting from mastitis. A review. *J. Dairy Sci.*, 53, 1151–1161.
- KÄSTLI, P., 1967. Definition of mastitis. *A. Bull. int. Dairy Fed.*, Part III, 1–5.
- KOHN, J., 1968. Cellulose acetate membrane (CAM) electrophoresis. In WILLIAMS, C. A. & CHASE, M. W. (Ed.), 1968. Immunology and Immunochemistry. Vol. II, 20–25. New York & London: Academic Press.
- MACLEOD, R., 1967. The preparation of plant viruses for use as antigens. In WILLIAMS, C. A. & CHASE, M. W. (Ed.), 1967. Immunology and Immunochemistry, Vol. I, 104–110. New York & London: Academic Press.
- MANCINI, G., YAERMAN, J. P., CARBONARA, A. O. & HEREMANS, J. F., 1963. A single radial-diffusion method for the immunological quantitation of proteins. *Prot. Biol. Fluids*, 11, 370–373.
- MORRIS, M. N. & HOBBS, W. B., 1971. Diagnosis of bovine mastitis by radial immunodiffusion. *Jl S. Afr. vet. med. Ass.*, 42, 143–147.
- ORNSTEIN, L., 1964. Disc Electrophoresis. I. Background and theory. *Ann. N.Y. Acad. Sci.*, 121, 321–349.
- PESKETT, G. R., 1932. Tests of abnormality of milk and of the udder. *Rep. natn. Inst. Res. Dairy for 1932*, 52–53.
- POLIS, D. B., SHMUKLER, H. W. & CUSTER, J. H., 1950. Isolation of crystalline albumin from milk. *J. Biochem.*, 187, 349–354.
- PRESCOTT, S. C. & BREED, R. S., 1910. The determination of the number of body cells in milk by a direct method. *J. infect. Dis.*, 7, 632–640.
- SCHALM, O. W. & LASMANIS, J., 1968. The leukocytes: Origin and function in mastitis. *J. Am. vet. med. Ass.*, 153, 1688–1694.
- SCHALM, O. W. & NORLANDER, D. O., 1957. Experiments and observations leading to the development of the California Mastitis Test. *J. Am. vet. med. Ass.*, 130, 199–204.
- SCHALM, O. W., CARROLL, E. J. & JAIN, N. C., 1971. Bovine mastitis. Philadelphia: Lea & Febiger.
- SHAH, P. C. & MORSE, G. E., 1964. Studies on albumin in "mastitic" milk. Factors affecting the presence of blood-serum albumin in "mastitic" milk. *Am. J. vet. Res.*, 25, 714–719.
- TOLLE, A., 1971. A monograph on bovine mastitis. *A. Bull. int. Dairy Fed.*, Part I, 1–23.
- TOLLE, A., ZEIDLER, H. & HEESCHEN, W., 1966. Ein Verfahren zur elektronische Zählung von Milchzellen. *Milchwissenschaft*, 21, 93–97.
- TOZER, B. T., 1967. Dissociation of antigen-antibody complexes in a salt-free medium with carbon dioxide: Preparation of specific precipitates for dissociation by carbon dioxide. In WILLIAMS, C. A. & CHASE, M. W. (Ed.), 1967. Methods of immunology and immunochemistry. Vol. 1, p. 349. New York & London: Academic Press.