IMMUNOLOGICAL DYNAMICS OF SPECIFIC ANTIBODY AND BACTERICIDAL ACTIVITY IN BLOOD SERUM AND MILK WHEY OF MASTITIC GOATS.

PS Gwakisa, G Killango, R Nkya and E Rugaimukamu, *Sokoine University of Agriculture, Faculty of Veterinary Medicine, P. O. Box 3019, Morogoro, Tanzania*

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SUMMARY

Agglutinating antibodies and bactericidal activity were assessed in blood serum and milk whey in relation to *Actinomyces pyogenes* in clinically mastitic and normal goats. Higher antibody response and bactericidal activity were observed in the sera of mastitic than normal goats. Milk whey was shown to have less agglutinating antibodies and essentially had no significant bactericidal activity to *A. pyogenes*. Determination of bacterial survival in the whey revealed that mastitic whey was capable of supporting bacterial replication better than normal whey. Bearing in mind that *A. pyogenes* frequently causes chronic or recurrent infections, these data suggest that antibody and the milk bactericidal mechanisms alone, may not be effective enough to prohibit udder infection by *A. pyogenes*.

INTRODUCTION

The recently increased popularity of the dairy goat requires more research inputs to generate information necessary for the effective control and therapy of caprine udder infections. In comparison to the extensive literature on bovine mastitis (Frost *et al.*, 1980; Mackie *et al.*, 1986; Yang *et al.*, 1988; etc), relatively little has been published on caprine mastitis, particularly on host protective mechanisms in relation to different mastitis pathogens. Due to the presence of a wide variety of potential udder pathogens and the ability of some of the pathogens to evade host protective mechanisms, assessment of components of the immune system is particularly important. Previous observations on the prevalence of different bacteria isolated from mastitic cases of goats in the University herd at Sokoine University of Agriculture, revealed *Actinomyces pyogenes* (formerly *Corynebacterium pyogenes*) as the most common mammary pathogen (Gwakisa, unpublished observations). Infections by this organism arise when the bacteria gain entrance to tissues as a result of various injuries and other infections caused by viruses, mycoplasmas and other bacteria.

Although *A. pyogenes* is frequently found in mixed infections, but on its own it also causes chronic abscessing mastitis, chronic suppurative pneumonia, septic arthritis and other conditions (Carter and Chengappa, 1991). The precise role of this organism in goat udders and the immune response it provokes have not been adequately described. Few reports have described the potential role of humoral

mediators and polymorphonuclear leukocytes (PMNL) in the development of the immune response in the bovines following infection with this organism (Watson, 1989). However, inspite of the presence of a number of antibacterial factors in the udder, chronic mastitis due to *A. pyogenes* and other pathogens is rather common (Husband, 1988; Dobrzanski and Yang, 1992). We here describe immunological dynamics of agglutinating antibodies and bactericidal activity in the blood serum and milk whey of mastitic and healthy goats in relation to *A. pyogenes*.

MATERIALS & METHODS

Animals

Crossbred dairy goats of a Norwegian landrace x Tanzanian local composition belonging to the Department of Animal Science in Sokoine University, were used in these studies. The goats were in their first to third lactation.

Mastitis Screening, Milk Sampling and Culture of Bacteria.

Twenty goats were screened by the California Mastitis Test (CMT). Milk samples (20 ml) from teats showing a positive CMT were collected aseptically at the beginning of milking. The samples were taken to the laboratory for culture work not later than 45 min after collection. Inoculations were done on sheep blood agar (SBA) and MacConkey agar for isolation of bacteria. Milk bacteriology was carried out following the recommendations of the International Dairy Federation (IDF,1981).

Preparation of Bacteria

A. pyogenes were maintained on SBA at 4°C. Forty eight hours prior to use in bactericidal assays, the bacteria were subcultured on SBA plates and incubated at 37°C. The bacteria were then harvested and washed twice in sterile phosphate buffered saline (PBS) before resuspension to the required final concentration of 10^8 ml^{-1}.

Preparation of A. pyogenes Antigen

A. pyogenes was grown onto SBA in Petri dishes for 48h at 37°C. The bacterial cells were harvested in 2 ml of nutrient broth (NB) and spread onto SBA prepared in large conical flasks. Following 48h of incubation at 37°C the bulk culture was harvested with glass beads in sterile PBS (pH 7.3), washed twice by centrifugation (6000 rpm /30 min) and resuspended in 20 ml PBS. The culture was tested for purity by inoculating a loopful of suspension onto SBA which was incubated at 37°C and examined 48h later. The bacterial cells were heat-killed and the suspension was kept in small aliquots at -20°C until use. A portion of the suspension was centrifuged (6000 rpm/30 min) to obtain soluble *A. pyogenes* antigen extract for passive haemagglutination assay.

Preparation of serum and whey samples

Blood and milk were collected from adult lactating goats whose mastitis status was established by results of the CMT. Five clinically mastitic and five apparently

healthy goats were used in all subsequent work. Whey was separated from the whole milk by centrifugation at 6000 rpm for 1 hour. Venous blood was collected into plain vacutainer tubes and allowed to clot and serum was separated by centrifugation (2500 rpm/15 min). The samples were filter-sterilized (0.2μm filters, Nalgene) and stored in small aliquots at -20°C until use. Prior to freezing, an aliquot of each sample was heat-inactivated (56°C/30 min).

Assay for agglutinating antibody

Antibody titres were determined by SAT and passive hemagglutination assay (PHA). For the PHA assay, sheep red blood cells (SRBC) collected in Alserver's solution (1:1) were washed twice in PBS (2500 rpm/15 min) and prepared to make a 1% solution. The SRBC were sensitized by incubation at 37°C for 1h with *A. pyogenes* antigen extract (1:1) in macrotitration plates. Two fold dilutions of serum and whey samples were dispensed into the wells containing the sensitized SRBC and the assay was incubated further for 2h at 37°C. Controls were included in which antigen-coated SRBC were tested for spontaneous agglutination.

Bactericidal assay

Fifty μl of *A. pyogenes* suspension containing $5x10^6$ bacterial cells was exposed to serum or milk whey which were used either neat or diluted 10X or 100X in nutrient broth containing 5% horse plasma (NB) to make a final volume of 400 μl. Immediately upon contact and subsequently after 1h, 2h, 3h and 6h of incubation at 37°C, 5μl was picked and diluted 1000X in NB. Earlier studies in our laboratory showed that after 6h of incubation most sera completely lost their bactericidal activity to a number of bacteria and logarithmic growth of these bacteria is observed (Gwakisa *et al.*, 1992). Fifty μl from this dilution was transferred into 20 ml of warm (55°C) blood agar, mixed thoroughly, and the mixture was poured into a sterile petri dish. A control dish containing untreated $5x10^6$ bacterial cells in blood agar with neither serum nor whey was set to check for nonspecific killing. After solidification the dishes were incubated at 37°C. Results were read after 24h-48h by counting individual hemolytic colony forming units (CFU) on a colony counter. Bacterial killing was calculated as a percentage of the difference between the number of bacteria in the original bacterial suspension and the number of CFU after incubation to the original bacterial suspension. Bacterial survival was expressed as a percentage of the direct count of CFU after 6h to the original bacterial suspension.

RESULTS

Agglutinating antibody response

Table 1 summarizes results of antibody titres in blood serum and milk whey of five mastitic and five non-mastitic goats. These results showed that mastitic goats had higher agglutinating antibody titre than non-mastitic goats. This was observed for antibody titres measured by either method, SAT or PHA. The data in Table 1 also show that higher antibody levels were detected in blood serum than

in milk whey.

Bactericidal activity

Results of this study showed that, the serum of both groups of goats, mastitic and non-mastitic, had bactericidal activity to *A. pyogenes in vitro*. As data in Table 2 show, the bactericidal activity was higher in mastitic animals than non-mastitic animals and further, this activity decreases in the course of incubation.

When bactericidal activity was determined every 3h for 24h, it was observed that this activity was not measurable beyond 6h of incubation. When bactericidal activity was measured in 10-fold dilutions of the serum samples (Table 3), results of these experiments showed that the bacterial killing strength in the sera of non-mastitic goats dropped by 49.2% when diluted 10 times and by 57.1% when diluted 100 times. The bacterial killing potency of sera from mastitic goats dropped by 21.4% and 31.9% when diluted 10 times and 100 times respectively.

In order to express the interaction between the serum bactericidal activity and the bacteria beyond 6h of incubation, we determined bacterial survival as described above. Intact and heat-inactivated sera and whey were used in these assays. The results are summarized in Table 4.

The nutrient broth medium control, in which neither serum nor whey was added supported only 25% of the original number of bacteria after 6h of incubation. Our results show that blood serum and milk whey from mastitic and non-mastitic goats, allow significant growth of *A. pyogenes* after 6h of incubation at 37°C. The ability to support bacterial survival was evidenced in the intact as well as heat-inactivated sera and whey. Thus, bacterial survival was increased by 44.8% in heat-inactivated serum, than in intact serum. Studies with the whey revealed that, before heat-inactivation, whey of mastitic goats supported bacterial growth by 52.8% more than whey of non-mastitic udders. This study further indicated that, heat-inactivated whey from non-mastitic and mastitic animals, supported growth of *A. pyogenes* better than intact whey by 12.4% and 23.7% respectively.

Table 1. Antibody titre to *A. pyogenes* in sera and whey of matistitic or non mastitic goats measured by SAT and PHA

Samples	Mean titre	
	SAT	PHA
Serum		
Mastitis +ve	1:64	1:96
Mastitis -ve	1:32	1:8
Whey		
Mastitis +ve	1:8	1:6
Mastitis -ve	1:2	0

Table 2: *In vitro* killing of *A. Pyogenes* by goat sera

Serum	% Killing after	
	1 hr	3 hr
Healthy	**78.9**	**46.7**
Mastitic	**93.5**	**55.1**

Table 3: Titration of bactericidal activity in mastitic or healthy goat sera

	% Killing after 3 h		
Serum	Neat	1:10	1:100
Healthy	46.7	23.7	20.0
Mastitic	55.1	43.3	37.5

Table 4: Bacterial survival after 6h of incubation in intact and heat-inactivated sera and whey.

Sample	% survival
Medium (Nutrient broth only)	25.0
Mastitic goat serum	
Intact serum	89.6
Heat-inactivated serum	162.5
Intact whey	
Non-mastitic goats	70.8
Mastitic goats	150.0
Heat-inactivated whey	
Non-mastitic goats	92.8
Mastitic goats	171.4

DISCUSSION

Our interest to this particular study arose, partly due to the prevailing void in the knowledge on the immune response to *A.pyogenes* during caprine mastitis. The results presented here allow several conclusions to be made.

Firstly, caution was exercised before selection of mastitis-positive animals, particularly in cases of conflicting CMT and bacterial culture results. One of the reasons for this caution is that negative culture results of CMT positive milk do not always rule out the possibility of presence of bacterial infection in the udder. Negative culture results of CMT positive milk may be due to poor growth of the bacteria outside the udder and to a greater extent, due to the small bacterial count in the sample as a result of ingestion and killing by phagocytic cells *in vitro*. Therefore the mastitis status referred to in this work, cites animals which showed positive reactions in the CMT and culture tests.

Secondly, the present work has shown that the titer of agglutinating antibodies to *A. pyogenes* was higher in mastitic goats than in non-mastitic animals. This observation allows one to conclude that the goat immune system is capable of mounting an antibody response to *A. pyogenes*, and the amount of antibody produced can be used to estimate the level of the immune response to this pathogen. The finding that a higher titre of specific antibody was detected in blood serum than in milk whey strongly indicates that besides the antibodies locally produced in the udder, a significant amount of the humoral mechanisms required for the udder immunity is transported from blood. Our findings are also supported by the knowledge that the goat udder synthesizes only IgA, whereas the bulk of the agglutinating antibodies (IgG and IgM) reach the udder from the blood

stream (Tizard, 1987). The reduced agglutinating antibody response in milk whey is also partly, due to the constant dilution of immunoglobulins in the udder brought about by the milking process.

Thirdly, the present study has demonstrated that *A. pyogenes* is susceptible to killing by blood serum from mastitic as well as non-mastitic goats. The serum bactericidal activity was higher in mastitic goats than in non-mastitic goats. It was possible to demonstrate that the bactericidal potency of the sera can be titrated, indicating that this activity can be used to discriminate between mastitic and non-mastitic animals. The increase of serum bactericidity with mastitis infection suggests that this activity is involved with the protective response to *A. pyogenes*. Altbough the role of bactericidal activity was not studied in relation to protection, but the results of this work and those reported by Kaartinen *et al.*, (1989) and Wijerwardana & Sutherland, (1990) strongly suggest that this immune mechanism is important in bacterial killing. Data obtained from this work has also shown that following prolonged incubation (6h and longer), the serum and whey samples lose their bactericidal activity thus allowing replication of the remaining bacteria. Mastitic whey supported replication of the bacteria to a greater extent than non-mastitic whey. The ability of mastitic milk whey to support increased replication rates better than non-mastitic whey may be due to the presence of increased amounts in them of key nutrients, such as heme iron and nitrogenous nutrients (Mattila *et al.*, 1984; Kaartinen and Sandholm, 1987). Milk from mastitic quarters contains various antibacterial factors including phagocytes, antibodies, complement, lysozyme, lactoferrin, and lactoperoxidase (Reiter, 1978). All antibacterial factors, except cells, remain present in whey. Inspite of the accumulation of these factors in milk whey, tbeir effect may be restricted to the first few hours of contact with the bacteria, when the hacteria are in their adaptation period (lag phase). This presumption seems to be legitimate with pathogenic bacteria, such as *A. pyogenes*, which are capable of causing chronic mastitis. We have also shown that removal of one of the antibacterial factors, complement, from the sera and whey, increases the ability of the samples to support replication rates of *A. pyogenes*. The most appealing explanation here seems to be that removal of complement totally shuts the bactericidal activity which is essentially an antibody-mediated response, operational via activation of the complement system (Wijerwardana & Sutherland, 1990).

In conclusion, this work has clearly demonstrated that antibodies as well as other antibacterial factors are important in killing of *A. pyogenes* in caprine mastitis. However, the antibacterial factors appear to have partial effectiveness, what was suggested by their loss of activity after 6h of incubation leading to multiplication of non killed bacteria. This observation may be used to explain the reason for chronic or recurrent infectlons of tbe udder caused by this pathogen.

REFERENCES

Carter, G.R. and Chengappa, M. M. (1991). Essentials of Veterinary

Bacteriology and Mycology. 4th Edn,. Philadelphia, Lea & Febiger 210-213.

Dobrzanski, M.J. and Yang, T.J. (1992). Systemic profiles of antigen - specific lymphocytes in animals chronically exposed to staphylococcus antigen in the mammary region. Comp. Immun. Microbiol. infect. Dis. 15:41-46.

Frost, A.J. Hill, A. W. and Brooker, B.E.. (1980). The early pathogenesis of bovine mastitis due to *Escherichia coli*. Proc. R. Soc. London, Sev. B, 209:431-439.

Gwakisa, P.S. and Minga, U.M. (1992). Humoral factors of natural resistance of *Bos indicus* cattle selected for antibody titre to *Brucella abortus*. Scand. J. Immunol. 36, Suppl. 11:99-102.

Husband, A.J. (1988). Migration and homing of lymphoid cells, Vols I and II. CRC Press, Boca Raton, Fla.

International Dairy Federation (IDF) (1981). Isolation and identification of mastitis bacteria. IDF Document 132:19-27.

Kaartinen, L. and Sandholm M. (1987). Regulation of plasmin activation in mastitic milk correlation with inflammatory markers and growth of *Streptococcus agalactia* J. Vet. Med. B. 34:42-50.

Kaartinen, L.; Ali-vehmas T.; Mattila T. & Sandholm M. (1989). Bacterial Growth in mastitic whey in relation to bacterial association with mastitis. Vet. Microbiology 21:153-163.

Mackie, D.P., Meneely, D.J., Pollock, D.A. and Logan, E.F. (1986). The loss of opsonic activity of bovine milk whey following depletion of IgA. Vet. Immunol. Immunopathol. 11:193-198.

Mattila, T., Maisi, P. and Sandholm, M. (1984). Haem compounds as bacterial growth promoters in whey: a possible application to bovine mastitis. Res. Vet. Sci. 36: 52-56.

Tizard, I. (1987). Veterinary Immunology. An introduction. 3rd edn. W.B. Saunders Company. Philadelphia 385.

Watson, E.D. (1989). Specific antibody in milk whey and phagocytosis of *Actinomyces pyogenes* by neutrophils *in vitro*. Res. Vet. Sci. 47:253-256.

Wijewardana, T.G. and Sutherland, A.D. (1990). Bactericidal activity in the sera of mice vaccinated with *Pasteurella multocida* type A. Veterinary Microbiology 24:55-62.

Young, T.J., Mather, J.F. and Rabinovsky, E.D. (1988). Changes in subpopulations of lymphocytes in peripheral blood, and supramammary and prescapular lymph nodes of cows with mastitis and normal cows. Vet. Immunol. Immunopathol. 18:279-285.