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Antibody class-specific immune responses in ovine milk measured by ELISA

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ABSTRACT

Diseases affecting mucosal surfaces, especially gastrointestinal disorders, cause high mortality rates in young domestic livestock. Hyperimmune milk products, using milk secretions from ruminants immunised systemically with enteric pathogens, have been shown to confer passive immunity against such disorders but the results are variable. To study the type and titre of immunoglobulin (Ig) classes produced in milk secretions after immunisation with gut pathogens, we have developed ELISAs for the estimation of ovine IgG and IgA antibodies directed against E. *coli*. Utilising an indirect ELISA technique, the method involved coating antigen onto microtitre plates then sequential addition of test sample dilutions (containing primary antibody), anti-class-specific antibody and anti-species horse radish peroxidase labelled antibody. Colorimetric end point detection employed 3,3',5,5'-tetramethylbenzidine and measurement of optical density at 450 nm. Colostrum sampled from normal untreated ewes (n=7) had low titres of both anti-E. *coli* IgG (0.87 ± 0.3; mean x10^{-3} ± s.e.m.) and IgA (0.25 ± 0.1), while colostrum from ewes (n=7) systemically immunised with E. *coli* during late pregnancy had high titres of anti-E. *coli* IgG (417.6 ± 169.4) but low titres of anti-E. *coli* IgA (3.2 ± 1.5). It can be seen that systemic immunisation with gut pathogens does not induce an IgA response in milk, although IgA is the predominant Ig of gut mucosal surfaces. Immunisation procedures to induce better IgA responses in ruminant milk secretions are currently being investigated which may result in more effective hyperimmune milk products.

Keywords: ELISA; immune responses; IgA; IgG; E. *coli* antibodies; hyperimmune milk.

INTRODUCTION

Diseases affecting mucosal surfaces, especially gastrointestinal disorders, remain a major cause of morbidity in young domestic livestock. Young animals in intensive rearing enterprises are particularly susceptible, for example scours in calves causes diarrhoea and dehydration and in severe cases death (Acres, 1985). Passive transfer of specific antibody, using hyperimmune milk secretions from ruminants immunised systemically with gut pathogens, has been shown to confer immunity to suckling neonatal calves and lambs (Snodgrass *et al.* 1980; Fahey *et al*, 1981; Crouch, 1985) but the results are variable (Wilson, 1980). In the primate and rodent, immunoglobulin A (IgA) is the major antibody of mucosal secretions and therefore of mammary secretions (Bienstock and Befus, 1980; Watson, 1980). However in the ruminant, the predominant antibody of colostrum and milk is IgG not IgA (Brandon *et al*, 1971), although some reports suggest that IgA would be more effective against gastrointestinal disorders (Mestecky, 1987).

In this paper, we report the development of an ELISA method for the estimation of class-specific E. *coli* antibodies and describe the milk IgG and IgA response of sheep systemically immunised in late pregnancy with this model gut pathogen.

MATERIALS AND METHODS

Assays

All samples and reagents were diluted with 0.01M phosphate buffered saline (pH 7.5) containing 0.05% v/v Tween-20 (PBS-T) and 1% w/v Bovine Serum Albumin (BSA; type A7030, Sigma Chem. Co., USA) and all washes were carried out by an automated plate washer (ELP-35, BioTek Instruments, USA) using PBS-T, unless otherwise stated.

ELISA plates (Maxisorp F-96 immunoplates, Nunc, Denmark) were coated with 100µl of E. *coli* antigen (Suvaxyn Maternafend-4; J & H Pacific Ltd, NZ) diluted 1:1000 in 0.05M carbonate buffer (pH 9.6), incubated overnight at 4°C and washed three times (3X). Remaining activated sites on immunoplates were blocked by incubating 2h at 22°C with 250µl PBS-T containing 1% w/v BSA. After washing plates 2X, 100µl of 10-fold serial dilutions of test samples (primary antibody) were added to duplicate wells. Plates were incubated 2h at 22°C then washed 3X. 100µl of second antibody consisting of heavy-chain specific rabbit anti-sheep IgG (RAS-IgG; diluted 1:600,000; prepared in house) and IgA (RAS-IgA; diluted 1:200,000; Bethyl Laboratories, USA) were added, each to separate plates. Plates were incubated overnight at 4°C, then washed 3X prior to the addition of 100µl of the enzyme conjugate, goat anti-rabbit Ig conjugated to horse radish peroxidase (GAR-HRP; diluted 1:8000; Dako, Denmark). After a 2h incubation at 22°C, the plates were washed 2X with PBS-T then 2X with PBS containing no Tween-20 and filled with 100µl of freshly prepared substrate solution. The substrate solution consisted of 0.1 g/l 3,3',5,5'-tetramethylbenzidine (TMB; Boehringer Mannheim, Germany) in 0.1M sodium acetate buffer (pH 5.5) containing 1.3 mmol/l hydrogen peroxide. Following a 30min incubation at 22°C, 50µl of stopping solution, 2M H_2SO_4, were added and the optical density (OD) was measured at 450nm by an automated plate reader (EL311s, BioTek Instruments, USA).

All ELISA plates were run with positive and negative control samples. The median OD between the maximum value for the positive control and the value for the negative

control was used as a reference point (reference OD) to determine test sample antibody titres. The reciprocal of the test sample dilution equivalent to the reference OD was given as the antibody titre

Animals

Fourteen pregnant Romney/Coopworth ewes were selected and randomly divided into two groups. Group 1 animals were immunised systemically at eight, four and one week prior to parturition. This involved deep muscle injection at 2 sites (1 ml/site) using E. *coli* antigen emulsified with Freund's Incomplete Adjuvant (FICA; Commonwealth Serum Laboratories, Australia; 1 part aqueous antigen to 3 parts FICA). Group 2 animals were untreated. After lambing, milk samples were collected on day 1 (birth), day 2 and day 5. Samples were initially centrifuged at 1200 rpm for 0.5h and the fat discarded. Supernatants then were centrifuged at 20,000 rpm for 1h and the aqueous portion stored at -20°C for antibody analysis.

RESULTS

Assays

Development procedures involved firstly determining approximate reagent concentrations by checkerboard titration using positive and negative control samples. Reagent volumes and dilutions were then optimised by varying one reagent while fixing the others until optimal OD signals were achieved, (e.g. 1-1.2 OD at maximum primary antibody binding and non-specific absorbance values of less than 0.1). Briefly, increasing the concentration of antigen or second antibody increased OD signals but also increased the non-specific signal. Conversely, decreasing these key reagents decreased the positive and negative control sample OD. Incubation times were also examined to check equilibrium rates for key reactions. Optimised dilution curves for a positive control sample in the IgG-ELISA and the IgA-ELISA are shown in Figure I. Assay precision was calculated from 10 repeat analyses of the positive control sample. The coefficient of variation was 8.0% for the IgG-ELISA and 7.8% for the IgA-ELISA.

Monospecificity of the RAS-IgA antisera was demonstrated by testing cross-reactivity with sheep IgG (Sigma, USA) in the IgA-ELISA. Using standard assay coating procedures, increasing concentrations of sheep IgG were coated onto ELISA plates. The plates were then incubated with RAS-IgA and developed with enzyme-conjugate as per the usual IgA-ELISA. Even at high levels of IgG, there was minimal increase of signal over background (Figure II). A similar cross-reactivity study with sheep IgA in the IgG-ELISA could not be undertaken due to the non-availability of pure sheep IgA. Monospecificity of the RAS-IgG and RAS-IgA antisera was verified in the ELISA by the observation that selected samples with a high titre for only one antibody class had undetectable antibody titres in the assay for the other antibody class.

Competition of IgG and IgA E. *coli* antibodies for antigen coated on the ELISA plates was tested by assaying samples with varying concentrations of IgG and IgA antibodies at a range of antigen coating concentrations. In this experiment, the ratio of IgG:IgA remained the same although titre values for IgG and IgA changed with different coating concentrations. In a second experiment, samples with varying concentrations of IgG and IgA antibodies were mixed and the antibody titres in the original samples and the combined sample measured. No significant difference between the measured titre of the combined sample and its calculated titre was observed (Table I).

FIGURE I: Estimation of IgG and IgA E. *coli* antibodies by ELISA.

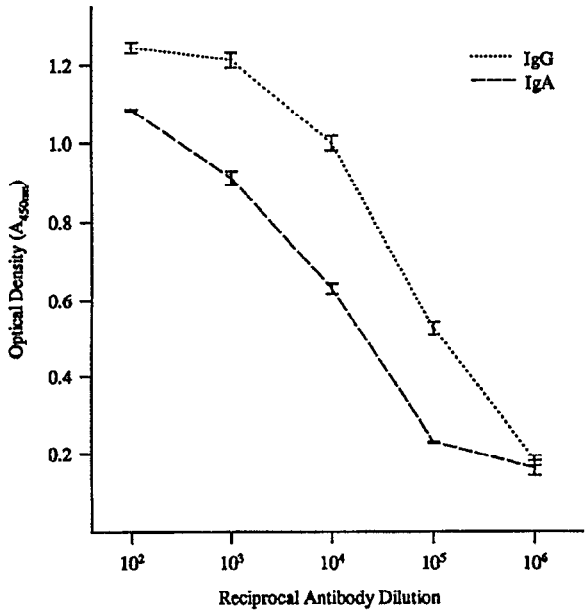
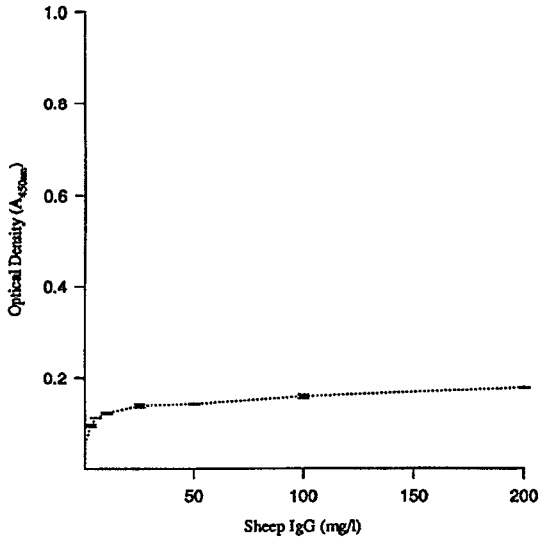


FIGURE II: Cross-reaction of IgG in the IgA-ELISA. Even at high concentrations of sheep IgG the optical density is less than 0.2, indicating minimal cross-reactivity of the RAS-IgA antisera with sheep IgG.



Animals

IgG and IgA E. *coli* antibody titres were determined by ELISA for the milk sampled from the animals at day 1, 2 and 5 post lambing. The group mean antibody titres are given in Table II. Day 1 colostrum samples from the untreated ewes (Group 2; n=7) had low titres of both anti-E. *coli* IgG

TABLE I: Competition of test sample antibody for antigen coated on ELISA plates. Samples with varying concentrations of IgG and IgA E. *coli* antibodies were mixed and the antibody titres in the original samples and the combined sample measured.

Sample	Anti-E. *coli* IgG titre	Anti-E. *coli* IgA titre
1	120,000	13,000
2	260,000	3,200
1 + 2 (measured)	190,000	7,500
1 + 2 (calculated)	190,000	8,000
3	15,000	19,000
4	560,000	4,000
3 + 4 (measured)	245,000	9,500
3 + 4 (calculated)	287,000	11,500

TABLE II: Class-specific antibody responses in milk samples from Group 1 ewes (systemically immunised with E. *coli* during late pregnancy) and Group 2 ewes (untreated).

Group	Day	Anti-E. *coli* IgG Titre	Anti-E. *coli* IgA Titre
1[a]	1	417.62 ± 169.41[b]	3.15 ± 1.49
	2	87.13 ± 33.14	0.45 ± 0.22
	3	28.15 ± 16.85	0.38 ± 0.26
2	1	0.87 ± 0.30	0.24 ± 0.11
	2	0.17 ± 0.14	0.07 ± 0.06
	3	0.01 ± 0.01	0.06 ± 0.04

[a] n=7[b] mean x10^{-3} ± s.e.m.

(0.87 ± 0.30; mean x10^{-3} ± s.e.m.) and IgA (0.24 ± 0.11). By contrast, day 1 samples from the systemically immunised ewes (Group 1; n=7) had high titres of anti-E. *coli* IgG (417.62 ± 169.41) but low titres of anti-E. *coli* IgA (3.15 ± 1.49). By day 5 all titres had fallen to less than 10% of day 1 samples.

DISCUSSION

This report describes ELISAs for the estimation of ovine IgG and IgA antibodies directed against E. *coli*. The accuracy of these tests is dependent on the quality of the reagents, the monospecificity of the class-specific antibodies being of prime importance (Clark and Engvall, 1980). We have confirmed the assay specificity by the observation that selected samples with a high titre of E. *coli* antibodies of one particular antibody class did not react in the ELISA of the other antibody class.

For our study, E. *coli* coated onto ELISA plates was used to capture all antibody classes specific for E. *coli*. This method has the potential for competition between antibody classes such that the abundance of one class of antibody could reduce the detectability of antibody of the other class (Van Zaane and Ijzerman, 1984; Hvatum *et al*, 1992). We were able to show that using our optimised ELISA conditions and at typical concentrations of test sample antibodies, we had no competition effects in this assay.

The ELISA method reported here has been used to assess the IgG and IgA responses of ewes to systemic immunisation in late pregnancy. The results have shown that this method of immunisation with gut pathogens is effective for inducing high titre antibodies of the IgG class but does not induce IgA antibodies even though IgA is the predominant immunoglobulin of gut mucosal surfaces.

Research effort is currently focused on the development of immunisation procedures specifically for the induction of mucosal IgA response which may result in more effective hyperimmune milk products for oral treatment.

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