CHAPTER VII

AN ATTEMPT TO DEVELOP AN ELISA TECHNIQUE FOR THE DETERMINATION OF THYROID STIMULATING HORMONE LEVELS IN CATTLE

INTRODUCTION

Various methods of assessing thyroid status in animals were reviewed in Chapter II. These covered measurements of iodide ions in blood, milk, feeds and urine as well as methods of assessing thyroid hormones in serum. Because of the complex mechanisms governing thyroid hormone regulation, there was a need to establish measurements of thyrotropin (thyroid stimulating hormone (TSH)), to increase diagnostic value of the thyroxin readings.

Over the past three decades (Mahan, 1998), the Enzyme Linked Immunosorbent Assays (ELISA) were developed as more powerful immunochemical techniques for a wide range of immunogenic substances (Anon, 1988). ELISAs are generally accepted as more accurate, faster and easier tests than other predecessor immunochemical tests (Anon, 1988). In the early stages, ELISAs relied on radio-active indicators in the quantitation of antigens and antibodies (Mahan, 1998). Safety considerations for laboratory operatives, hazards posed by disposal of radioactive waste, the short shelf life of radio-labelled reagents and the need for enhanced speed to obtain results, led to a search for safer and environmentally more friendly approaches in diagnosis. Many ELISA kits now utilise chromogen based indicator systems (Crowther, 1995). While ELISA test kits for Thyroid Stimulating Hormone (TSH) levels are well developed for humans and canines (Immunotech, 1991; DPC 1995), extensive literature search in

this study did not show this to be the case for bovines and caprines. Documented immunoassays used in this thesis for assaying T_3 and T_4 were radio-immuno assays. The ultimate test of choice would have been an ELISA; for example, a sandwich ELISA format that is generally considered most useful for quantitative antigen detection (Anon, 1988). The procedure is quick, accurate, reliable, specific, sensitive and can be used to determine levels of concentrations of most protein antigens. An additional advantage is its potential to handle large numbers of samples, making it ideal for population diagnostics. The TSH ELISA tests in use for humans and similarly for canines, are sandwich kit-based tests that utilise non-isotopic chromogens rather than radioactivity for quantitation readings (Immunotech, 1991; DPC, 1995). No TSH ELISA procedure could be identified that has been attempted for cattle.

.

AIM

To develop through adaptation, an ELISA test for the measurement of serum TSH levels in cattle.

MATERIALS AND METHODS

Lists of materials required for the tests and step by step procedures are given in APPENDIX I.

Preparation of TSH-Freunds adjuvant for immunising goats:

Column chromatography purified commercial bovine thyroid stimulating hormone (Reference AFP-3950B), obtained from the National Hormone and Pituitary Programme (NHPP), Torrance, California, USA was thawed and a 1ml aliquot of it

mixed with 1ml of commercial Freund's complete adjuvant (FCA) from Sigma Immuno Chemicals® St Louis, Minnesota, USA. The combination was aspirated into a 10ml hypodermic syringe. A second syringe was conjoined to this first one by a 16-gauge syringe connector and the TSH and adjuvant mixture was then forcefully squeezed to and fro the connector for about 300 times for more thorough mixing. During the mixing process, due care was taken not to denature the TSH by excessive heat generated during emulsification. Therefore every now and then, the mixture was kept cool by burying it under ice. The mixture was considered ready for inoculation when it became completely emulsified. The same procedure was used in making the second and third TSH preparations. The latter preparations used incomplete Freund's adjuvant (FA).

Preparation of Goat anti-bovine TSH serum

Two castrated Boer goats, (VR5349 and VR5360), aged 9 months, from Mazowe Field Station were identified for production of polyclonal antiserum against TSH. The two castrated males were brought to the Central Veterinary Laboratories in Harare and housed in animal cubicles for four weeks to stabilise them under confinement before any work was done on them. They were de-wormed and treated against mange during this period. A pre-inoculation blood sample for serum was collected from them and then the two goats were each inoculated subcutaneously in the neck region with 2 ml of the Freund's adjuvanted thyroid stimulating hormone. Four weeks later, a booster was given to the goats, at a different injection site, but still in the neck region. Two weeks after this booster, blood was collected from the goats for serological analysis of antibody response to the bovine thyroid stimulating hormone. Collection of blood for this purpose was repeated at two-week intervals

over 12 weeks to produce the post-exposure sera, later used as the test sera in evaluating various ELISA techniques.

Preliminary steps to determine reactivity of the reagents to be evaluated

a) Indirect ELISA

An indirect test format was attempted to determine the extent to which goats develop antibodies against bovine TSH, detectable in an immunological reaction as well as the reactivity of various reagents at different dilutions. This further step was taken to indicate reactivity of the second antibody system, the goat anti-bovine TSH antibody as a candidate for the sandwich ELISA test for bovine TSH.

First, a checkerboard titration reaction was conducted to determine the optimal working dilutions of the various components of the test. The conditions so determined for antibody, antigen and conjugate were used in all the test formats that followed.

Procedures for making the solutions and reagents used in the test are outlined in APPENDIX I.

Nunc maxisorp microtitre plates were coated with 100 µl per well of purified bovine TSH in carbonate buffer of pH 9.6, at concentrations of 2 µg and 4 µg selected from the checkerboard titration. The coated plates were incubated at 4^0C overnight. Blocking was achieved with 200 µl per well of 3% blocking solution, and incubated at 37^0C. The plates were washed four times, each time with 200µl per well of wash buffer solution, to remove excess TSH. Pre-exposure and post exposure goat sera were added at 100µl per well as described in the various tables of results. The plates were incubated for an hour at 37^0C, after which they were washed four times with wash buffer. Horse-radish peroxidase-conjugated anti-goat IgG from Kirkegaard

Perry Laboratory (KPL) was added at 100 µl per well, followed by incubation for an hour at 37^0C. The plates were then washed four times with 200 µl per well of wash buffer, after which 2,2'-azinobis-bis(3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt (ABTS), a chromogenic substrate in sodium citrate buffer, was added. The plates were incubated for 20 minutes at room temperature and the reaction was stopped with addition of 100 µl of 2 molar sulphuric acid. Optical density readings were taken in an ELISA plate reader at 405nm. Unreacted pre-inoculation and post inoculation goat sera on their own or as mixtures with antigen (TSH, LH (NHPP reference AFP11743B), FSH (NHPP reference AFP5332B); with conjugate only or with substrate only, provided the test control readings.

Optimisation of the reaction format

With prior knowledge (Parlow, 1999) that an immunonological reaction of ruminant TSH was likely to suffer from cross-reaction with Follicle stimulating hormone (FSH) and Luteinising hormone (LH), the indirect format described above was assessed for the reaction of the test sera with FSH, LH, a combination of the two as well as with TSH as the plate coating antigens. Wells with TSH only, buffer only, test sera with FSH, test sera with LH, test sera with TSH and with conjugate only, formed the controls to provide the background readings.

Pre-adsorption procedure

Controlling for the effects of cross-reaction with FSH and LH was performed in two ways. One way was to pre-react test sera on the various cross-reacting hormones, separately and in combinations in a separate plate. These pre-reacted sera were then

transferred to a TSH coated plate for the test. This approach was used for the indirect test format where the antigen was on the solid phase (APPENDIX I).

First, a plate was coated with 100µl per well of 2µg each of FSH, LH; FSH+LH and TSH. Following overnight incubation at 4^0 C, plates were washed 5 times with PBS. Blocking was achieved with 200ml of 3% skimmed milk, and incubated for an hour at 37^0 C. Test sera were diluted to 1:100; 1:300 and 1:900 in 3% skimmed milk and added to the plates at 100µl per well. The plates were incubated at 37^0 C for an hour. In the second stage, 100µl of the pre-adsorbed serum from the plates were then transferred to a TSH coated plate for the indirect ELISA test as described above. Control wells had sera adsorbed in TSH, FSH; FSH+LH and LH but in wells not coated with TSH; conjugate only, buffer only and the hormones on their own.

The second way was used for the sandwich procedure in which the first antibody on the solid phase, was pre-reacted with the various hormones. TSH or test serum was then added, followed by the second antibody, then the conjugate and substrate (APPENDIX I).

b) The sandwich ELISA procedure

With the NHPP rabbit anti-bovine TSH antibody (Reference AFP284246Rb), and having established that the goat anti-bovine TSH sera was reacting with bovine TSH, these were evaluated in a sandwich format. The intention of the sandwich procedure was to detect TSH in bovine sera collected in the field survey (Chapter III). The two antibody systems: Rabbit and Goat anti-bovine TSH had been confirmed for reactivity in preceding sections.

The procedure was first evaluated for cross-reactions with other hormones namely; FSH and LH. The first stage was to coat the plate with rabbit-anti-bovine TSH serum diluted in coating buffer to 1:100 at 100µl per well. The plate was incubated at 4^0 C overnight and then washed four times in PBS/0.1% tween 20. The plate was blocked with 200µl per well of skimmed milk in PBS/0.1% tween 20 and incubated at 37^0 C for an hour, followed by washing four times in PBS/0.1% tween 20. To each well was added 100 microlitres of FSH, LH and TSH at quantities of 2µg and 4µg diluted in 3% skimmed milk in PBS/0.1% tween 20. The FSH and LH were used to pre-adsorb the first antibody to minimise the effects of cross-reaction. The plate was incubated at 37^0C for an hour and then washed four times in PBS/0.1% Tween 20. Post-inoculation goat sera as the second antibody was then added at 100µl per well diluted in 1% skimmed milk/PBS/0.1% tween 20, at 1:100 at 100µl per well. The plate was incubated at 37^0 C for an hour and washed 4 times in PBS/0.1% tween 20. Horse-radish peroxidase labelled anti-goat IgG diluted 1:2000 in 1% skimmed milk/PBS/0.1% tween 20 was added at 100µl per well. The plate was incubated for an hour at 37^0C and washed 4 times again as above in wash buffer. Last, 100µl of ABTS substrate were added per well followed by incubation on a plate shaker at room temperature for 20 minutes. The reaction was stopped by adding 2 molar sulphuric acid at 100 µl per well. Optical density readings were taken at 405nm. Control wells contained the various reagents separately or in combination but without the test hormone. The procedure was also done for each hormone using 4µg of each of FSH, LH and TSH to check the reaction due to each hormone separately.

d) The checkerboard titration for test control

Because of background reactivity detected in the indirect ELISA and in the control wells of the sandwich procedure described above, the latter was subjected to a checkerboard titration to try and identify a suitable control system with sufficient discriminatory power against the test sera. The aim of this was to find a negative control with low background reaction. Foetal bovine serum and normal laboratory bovine serum were thus tested.

RESULTS

There was significant difference in reaction between goat sera, before and after inoculation with TSH. The reactivity was much higher after inoculation. Although there was also some background reaction in the in all control wells, this was fairly low and the main reaction was therefore reflecting the presence of anti-TSH antibodies in the pre-immunised goat serum, but not to the same extent as in the post immunised goat sera. Dilution of conjugate only marginally reduced the OD readings at all dilutions of serum and antigen levels (Table 7.1).

Table 7.1: Evaluation of an ELISA for bovine TSH by the indirect reaction format, 2000

Antigen concentration: 2µg/ml						
Serum dilution: 1:50 – OD Readings						
Conjugate dilution:	1:250		1:500		1:1000	
	Pre	Post	Pre	Post	Pre	Post
Goat #5349	0.387	1.441	0.259	1.307	0.183	1.109
#5460	0.557	1.405	0.439	1.246	0.279	1.080
Serum dilution: 1:100– OD Readings						
Goat #5349	0.238	1.274	0.166	1.112	0.126	0.949
#5360	0.318	1.236	0.237	1.114	0.168	0.917
Antigen concentration: 4µg/ml						
Serum dilution: 1:50						
Conjugate dilution:	1:250		1:500		1:1000	
	Pre	Post	Pre	Post	Pre	Post
Goat #5349	0.325	1.467	0.247	1.341	0.174	1.168
#5360	0.590	1.419	0.504	1.286	0.376	1.135
Serum dilution 1: 100						
Goat #5349	0.237	1.352	0.160	1.248	0.117	0.998
#5360	0.369	1.270	0.302	1.177	0.221	0.950

CONTROLS								
	Antigen		Conjugate		Substrate		Serum	
	2µg/ml	4µg/ml	2µg/ml	4µg/ml	2µg/ml	4µg/ml	2µg/ml	4µg/ml
Goat # 5349 – Pre	0.004	0.002	0.010	0.009	0.008	0.010	0.005	0.004
Post	0.008	0.005	0.003	0.004	0.005	0.007	0.007	0.001
Goat # 5360 – Pre	0.003	0.000	0.008	0.005	0.008	0.009	0.003	0.001
Post	0.010	0.009	0.002	0.003	0.001	0.002	0.008	0.005

Post inoculation goat serum gave higher OD's than pre-inoculation goat serum on all three hormones (FSH, LH, TSH). Control wells to which only sera preadsorbed to FSH, LH, TSH and a mixture of FSH and LH were added gave higher OD readings than those containing only conjugate or only hormone. Pre-adsorption marginally raised the OD background readings (Table 7.2). Attempts to remove cross reactivity with FSH and LH therefore did not improve the assay's specificity for TSH detection.

Table 7.2: Evaluation of the indirect TSH ELISA test controlling for the effects of FSH and LH by adsorption, 2000

Animal		Adsorbed in FSH	Adsorbed in LH	Adsorbed in TSH	Adsorbed in FSH + LH	TSH only	TSH only	TSH + buffer	*Buffer only*
5360	Pre-sera	0.383	0.253	0.216	0.263	0.087	0.085	0.071	*0.069*
	Post sera	1.212	1.304	1.134	1.569	0.069	0.064	0.056	*0.056*
5349	Pre-sera	0.140	0.148	0.128	0.134	0.063	0.062		
	Post sera	1.520	1.613	1.130	1.454	0.071	0.066		
		Only test sera adsorbed in FSH	Only test sera adsorbed in LH only	Only test sera adsorbed in TSH only	Only test sera adsorbed in FSH + LH	Conj ugate only	Conj ugate only		
5360	Pre-sera	0.100	0.108	0.110	0.112	0.075	0.059		
	Post sera	0.135	0.092	0.104	0.110	0.067	0.066		
5349	Pre-sera	0.138	0.108	0.102	0.106	0.057	0.061		
	Post sera	0.131	0.115	0.114	0.100	0.076	0.073		

Conjugate: Anti-Goat serum labelled with horse-radish peroxidase 1:2000

Substrate: ABTS (KPL)

Plates coated with 2µg TSH

The results of the reactivity of pre- and post- inoculation goat sera to the three hormones, TSH, LH and FSH indicated that reactions to each of the three hormones were relatively high. Increasing antigen levels from 2µg to 4µg raised the reactivity only marginally. Control wells with sera only gave significant background reactions exceeding 0.2 versus control readings in wells containing only hormones (Table 7.3). The test format was therefore recognising some reaction even in serum that had not been presented to antigen.

Table 7.3: Indirect ELISA for evaluating pre- and post- inoculation sera against TSH, FSH and LH antigens, 2000.

	Animal 5360	5349	5360	5349	5360	5349
	TSH 2µg	TSH 2µg	TSH 4µg	TSH 4µg	Sera only	Sera only
Pre-sera	0.587	0.276	0.728	0.264	0.236	0.284
Post sera	2.359	2.384	2.596	2.597	0.246	0.195
	FSH 2µg	FSH 2µg	FSH 4µg	FSH 4µg	Sera only	Sera only
Pre-sera	0.204	0.201	0.210	0.269	0.253	0.297
Post sera	2.013	1.780	2.043	1.929	0.323	0.271
	LH 2µg	LH 2µg	LH 4µg	LH 4µg	Sera only	Sera only
Pre-sera	0.217	0.239	0.211	0.234	0.258	0.290
Post sera	1.476	2.023	1.647	2.250	0.311	0.270
	TSH 2µg	TSH2µg	LH 4µg	LH 4µg	Buffer only	Buffer only
	0.075	0.061	0.073	0.092	0.077	0.071
	FSH 2µg	FSH 2µg	Conjugate only	Conjugate only	Buffer only	Buffer only
	0.094	0.207	0.091	0.081	0.091	0.075

Conjugate: antigoat IgG-HRP 1:2000

Pre- inoculation goat serum consistently contained lower hormone levels than post-inoculation goat serum but the reactions were similar against all the hormones. There

were no zero TSH levels in all the pre- inoculation sera and this indicated the presence of endogenous hormone.

Table 7.4: Indirect ELISA for examining the cross.reactions of Pre- and Post-inoculation sera following pre-adsorption with FSH, LH and TSH on coated plates, 2000.

		Serum pre-adsorbed in FSH (2µg)			Serum pre-adsorbed in LH (2µg)			Serum pre-adsorbed in TSH (2µg)		
Animal identity	Serum dilution	1:100	1:300	1:900	1:100	1:300	1:900	1:100	1:300	1:900
5360	Pre-sera	0.280	0.154	0.107	0.299	0.150	0.110	0.265	0.175	0.105
	Post sera	2.356	0.133	0.457	2.304	1.226	0.115	1.917	0.800	0.309
5349	Pre-sera	0.147	0.159	0.062	0.133	0.091	0.077	0.133	0.157	0.075
	Post sera	1.946	1.097	0.442	2.006	1.106	0.444	1.556	0.761	0.271
		Sera adsorbed in TSH only	Sera adsorbed in FSH only	Sera adsorbed in LH only						
5360	Pre-sera	0.099	0.080	0.088						
	Post sera	0.115	0.103	0.111						
5349	Pre-sera	0.075	0.052	0.056						
	Post sera	0.076	0.065	0.073						

Conjugate: anti goat IgG- HRP diluted 1:2000 in 1% skimmed milk-PBS in 0.1% Tween

Serum: Pre- and post inoculation goat serum in 3% milk PBS-Tween

Diluting the goat serum reduced the OD readings but did not remove the cross-reactions with LH and FSH. Unreacted sera gave significant background reactions in

comparison with readings obtained for wells containing unreacted hormone only or unreacted conjugate only (Table 7.4)

Optical density readings of the sandwich ELISA procedure for anti-TSH reactivity in pre- and post- inoculation goat sera having blocked out TSH, FSH and LH indicated that both pre and post- inoculation goat sera did not change (Table 7.4). The test therefore fails to sufficiently distinguish between TSH and the two gonadotrophins (TSH and LH). Serum dilution at 1:100 gave the highest reading in post-inoculation serum.

Table 7.5: Sandwich ELISA for TSH antibody levels in goat sera, checking the effect of cross-reaction to FSH and LH, 2000.

Animal Identity	ELISA ANTIGEN	TSH4µg	TSH 4µg	TSH 4µg	TSH 4µg No conjugate	No TSH	No TSH
5360	Pre-sera	0.776	0.839	0.899	0.069	0.934	1.023
	Post sera	1.085	0.970	0.943	0.059	0.601	0.600
	ELISA ANTIGEN	FSH4µg	FSH4µg	FSH4µg	FSH4µg No conjugate	No FSH	No FSH
5360	Pre-sera	0.536	0.810	0.682	0.056	0.632	0.653
	Post sera	0.660	1.001	0.826	0.057	0.618	0.662
	ELISA ANTIGEN	LH 4µg	LH 4µg	LH 4µg	LH 4µg No conjugate	No FSH	No FSH
5360	Pre-sera	0.476	0.795	0.688	0.055	0.527	0.591
	Post sera	0.505	0.772	0.606	0.058	0.530	0.441

Solid phase antibody: rabbit anti-bovine TSH antiserum 1:100

Second antibody: Pre- inoculation sera 5360 diluted to 1:100, Post inoculation sera 5360 diluted to 1:100

Conjugate: Anti-goat IgG – Horse radish peroxidase labelled 1:2000

Substrate: ABTS substrate system (KPL)

Significantly high reactions for FSH and LH were still obtained as with TSH in a sandwich format (Table 7.5). The reaction was therefore not unique to TSH. Again control wells which had hormone but excluded conjugate recorded ODs less than 0.06, whereas when they contained serum and nothing else, the ODs were higher than 0.50 (Table 7.5).

Table 7.6: OD readings of a checkerboard Titration of bovine TSH and pre-inoculation goat sera: Determination of dilution levels of test sera and TSH levels using Sandwich ELISA, 2000.

Rabbit anti-bovine TSHanti-serum→ ↓Pre-inoculation goat sera

Serum dilution	1:100	1:200	1:400	1:800	1:600	1:3200	1:6400	1:12800
1:100	1.256	1.375	1.285	1.409	1.379	1.338	1.258	1.386
1:200	1.092	1.129	1.204	1.255	1.170	1.250	1.241	1.347
1:400	1.441	1.119	1.115	1.090	1.199	1.219	1.077	1.163
1:800	0.900	0.778	0.757	0.735	0.759	0.783	0.908	0.863
1:1600	0.570	0.594	0.574	0.470	0.597	0.567	0.626	0.592
1:3200	0.417	0.411	0.427	0.447	0.425	0.641	0.484	0.458
1:6400	0.391	0.347	0.335	0.352	0.374	0.294	0.344	0.355
1:12800	0.413	0.382	0.316	0.337	0.405	0.347	0.352	0.380

Conjugate: anti-goat IgG – HRP 1:2000

Substrate: ABTS (KPL)

Antigen: 2microgram TSH per well

Background reaction was not removed by dilution of either conjugate or unexposed goat serum. The reaction format is therefore recognising some complex. However, while goat serum seemed to respond better to dilution, the rabbit anti bovine TSH antiserum did not.

Table 7.7: TSH SANDWICH ELISA: OD readings of a checkerboard titration of bovine TSH and foetal calf serum to check for non-specific reactions using fetal calf serum, 2000.

Rabbit anti-bovine TSH anti-serum→↓Foetal calf serum

Serum dilutions	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800
1:100	1.374	1.416	1.483	1.481	1.585	1.179	1.564	1.551
1:200	1.160	1.216	1.205	1.238	1.313	1.234	1.246	1.300
1:400	0.913	0.874	1.015	0.981	1.081	1.002	1.001	1.025
1:800	0.492	0.645	0.615	0.681	0.763	0.671	0.709	0.726
1:1600	0.395	0.463	0.541	0.480	0.459	0.632	0.491	0.513
1:3200	0.350	0.314	0.397	0.363	0.355	0.633	0.373	0.367
1:6400	0.301	0.281	0.334	0.298	0.320	0.481	0.314	0.319
1:12800	0.305	0.377	0.297	0.304	0.295	0.337	0.570	0.340

Conjugate: anti-goat IgG– HRP 1:2000

Substrate: ABTS (KPL)

Antigen: 2 microgram TSH per well

Background reaction still occurred with foetal calf serum. Rabbit anti-bovine TSH antiserum again does not seem to respond well to dilution in comparison with the foetal calf serum.

Table 7.8: OD readings of a checkerboard titration of Antibovine TSH against Normal Bovine Serum (NBS). Checking for negative bovine serum

Rabbit anti-bovine TSH anti-serum→↓Normal Bovine serum

Serum dilution	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:1280
1:100	1.173	1.254	1.495	1.431	1.616	1.611	1.643	1.352
1:200	1.099	1.197	1.153	1.462	1.403	1.411	1.460	1.496
1:400	0.971	0.907	1.127	0.854	1.115	1.117	1.177	1.164
1:800	0.661	0.819	0.778	0.597	0.831	0.837	0.864	0.748
1:1600	0.494	0.550	0.610	0.597	0.485	0.596	0.499	0.539
1:3200	0.416	0.346	0.439	0.381	0.432	0.379	0.434	0.379
1:6400	0.348	0.346	0.403	0.346	0.378	0.292	0.378	0.386
1:12800	0.259	0.371	0.347	0.360	0.349	0.339	0.353	0.336

Conjugate: anti-goat IgG – HRP 1:2000

Substrate: ABTS (KPL)

Antigen: 2microgram TSH per well

Background reaction reflects that the test picks up TSH in normal bovine serum. The pattern of reaction is once again similar to that obtaining in Tables 7.6 and 7.7.

The checkerboard titration for optimising the sandwich ELISA procedure indicated that high background OD's on all three sera occurred, even at low dilutions. In particular, while the three negative control sera responded to dilution the rabbit anti-bovine TSH antiserum is not a sensitive reagent as an antibody in a sandwich format as it compounds the background reaction by seemingly reacting with something in the test that dilution does not seem to influence easily. The problem of cross-reactions

among gonadotrophic hormones as well as the background reactions in control wells meant that the sandwich format as described here could not be relied upon to test field sera. The rabbit anti-bovine TSH antiserum like the bovine TSH were a donation for research by the NHPP, USA. There was no other reagent to rely on at the time.

DISCUSSION

Increases in optical density OD readings in post-inoculation serum samples illustrate that there is room for the development of an ELISA test for TSH to determine various levels of the hormone. TSH is however expected to be present in the blood of mammals as a naturally occuring endogenous hormone. Its presence in pre-inoculation sera is therefore explicable. It is expected to complicate the readings since its levels may fluctuate in response to homeostatic demands on the pituitary, in reaction to variations in thyroid hormone levels. Information sought was about variations in levels normal to given situations. This information would help in the interpretation of laboratory findings with respect to health and production performance. Therefore it will be necessary to find ways of blocking or accounting for background reactions in order to have explicable meanings for test samples. This relates to the problem due to background reactions in controls as well as the problem of cross-reactivity with other hormones which reduces specificity of the test.

It was also noted that the test was sensitive to changes in dilution of both serum and antibody. This suggests that despite the lack of negative controls, optimal test conditions can still be defined and varying levels of hormone concentration can be detected following exhaustive test validation and modification. It was noted for example that conjugate (HRP-labelled anti-goat IgG) dilution of 1:2000 is expected to yield reasonable discrimination between exposed and non-exposed sera. Preliminary

procedures using the indirect format indicated that a test serum dilution of 1:100 can detect up to 2 µg of antigen fairly distinctly. The test is also expected to perform best at serum dilutions at least 1:100, conjugate dilutions of at least 1:1000, and antigen concentration of 2 µg/ml. Determination of a cut-off point or construction of a standard curve from which to take readings will be important.

In the test, exposure to extraneous TSH increased the levels of reactivity by test sera, to all three pituitary hormones (FSH, LH and TSH) by almost equal magnitude resulting in consistent cross-reaction amongst the three hormones (Table 7.2). This implied that the test was identifying a component common to all three. In this event, the effect is interactive but according to literature (Immunotech, 1991, DPC, 1995; Borger and Davis, 1974), it should be possible to remove the effects due to the others if specific means can be found. This is from the point of view that the mechanisms for the stimulation of production of these hormones as well as their functions, are specific and it is highly unlikely, except where there are tumours, that the different pathways can have a common effect simultaneously for all hormones interactively. Literature provided with the NHPP reagents pointed out that cross-reactions can be expected with other pituitary hormones, LH, FSH and choreonic gonadotrophin due to similarities in their alpha sub-units (DPC, 1995).

It is therefore not possible to use this format specifically for any one of the three hormones and measures to remove this cross-reactivity are therefore essential. Contrary to findings by Borger and Davis, (1974), blocking of LH and FSH prior to reaction, by adsorption, did not significantly reduce cross-reactivity in polyclonal sera in the present study. It will therefore be necessary to test alternative blocking mechanisms such as the use of sepharose immuno-adsorption and beta specific radio-iodinated TSH reagents (Borger and Davis, 1974).

It has been suggested that the ß subunit renders the TSH molecule more unique in giving the individual pituitary hormones their biological and immunological qualities. That subunit might therefore have been more discriminating (Immunotech, 1991). It is therefore necessary that the two antibodies in a sandwich format, bind discretely to non-overlapping epitopes on the antigen. Alternatively, polyclonal antibodies that are affinity-purified (Anon, 1988) could be more effective in discriminating cross-reacting elements. This suggests greater epitope targetting, requiring the generation and use of specific monoclonal antibodies for the ß epitope. This possibly explains the success of the human and canine TSH sandwich ELISA tests, both of which are based on a monoclonal antibody-conjugate (Immunotech, 1991; DPC, 1995). The sandwich ELISA method is regarded as the best to determine antigen concentration in unknown samples containing unpurified antigen (Anon, 1988). In the present study, the goat antibodies were polyclonal and did not possess the qualities to permit accurate differentiation. The rabbit anti-bovine antiserum was also neither affinity-purified nor was it monoclonal (Parlow, 1999). In earlier preliminary laboratory trials preceding this study at the CVL, Harare, using the monoclonal antibody based human ELISA kit on bovine samples however, the results were disappointing suggesting that the bovine kit would need to have different and bovine specific reagents. Ferguson (1994) also found that a using human TSH assay for canines was not successful. This is despite assertions that the alpha epitope of the TSH molecule is expected to cause cross-reactions with other pituitary hormones only within a species (DPC, 1995). Species factors are therefore as important as the purity of reagents. Extensive reagent search had also failed to yield a source of epitope-specific monoclonal antibodies. These underlying aspects could not be addressed due to the scope of this study that was

limited by cost and available capacity. More literature search, time and effort are therefore still needed to develop this bovine TSH-ELISA.

CONCLUSION

A sandwich ELISA test format can work in the detection in serum, of thyrotropin (Thyroid Stimulating Hormone or TSH) in cattle. However, cross-reactions with other gonadotrophic hormones interfere with specificity of the test. Attempts to remove the cross-reaction through pre-adsorption were not effective. Before this sandwich ELISA protocol can be routinely applied, various alternatives to this problem such as use of beta specific monoclonal antisera or the use of immuno-adsorption will need to be investigated. Secondly, means are required to remove background reaction affecting control reagents including control sera. It was therefore decided that the test as outlined here could not be used in testing bovine sera collected in a field cross-sectional study described in Chapter III.