APPENDIX I

MATERIALS AND PROCEDURES FOR THE ELISA PROCEDURES

A) Materials and reagents

Na_2CO_3
$NaHCO_3$
$Na_2HPO_4.2H_2O$
Na_2HPO_4
Citric Acid.H_2O
30% H_2O_2
NaCl
KCl
KH_2PO_4.
ABTS Sigma catalogue number A-1888 2 grams
Phosphate buffered saline (PBS)
ELISA plates-nunc maxisorb
Micro-titre plate sealers
ELISA reader
Skimmed (non-fat) milk, Dairi-lite Dairiboard Zimbabwe limited
Affinity purified Horse Radish Peroxidase labeled anti-goat IgG (High and Low)
Kirkgaard-Perry Laboratories, 2 Cessna Court, Gaithesburg, MD 20879, USA

Solutions

1. Coating Buffer
   50 mM Na_2CO_3 -- 0.155 grams
   35 mM $NaHCO_3$ – 0.293 grams

Dissolve in 100 ml distilled water
Check pH to remain at 9.6
Fresh buffer to be prepared each time plates are coated

2. Substrate buffer stock solution
   Solution A (0.2 M Phosphate buffer)
   Na_2HPO_4. $.2H_2O$ - 8.9 grams
   Dissolve in 250ml of distilled water

   Solution B (0.1 M Citric Acid
   Citric Acid.H_2O - 5.25 grams
   Dissolve in 250 ml of distilled water

3 Substrate solution
   -Mix equal parts of solution A and Solution B to make the substrate buffer (minimum 10 ml each to make 20 ml)
   -Measure the pH and maintain at pH 5.0
   - Dissolve a 10 mg ABTS tablet or 10 mg ABTS powder for every 20 ml of freshly prepared substrate buffer

- Scale up the amount of substrate depending on the number of ELISA plates
- Add 5 microlitres of 30% H_2O_2 for every 20 ml of substrate just before dispensing substrate onto plates

4 Blocking and diluting solution
0.1M Phosphate Buffered Saline (PBS)
0.1% Tween 20
1% Skimmed milk

5 Wash Solution
0.1M PBS
0.1% Tween 20

6 PBS
NaCl 8 grams
KCl 0.2 grams
Na_2HPO_4. - 1.44 grams
KH_2PO_4. - 0.24 grams
One litre distilled water
Add 0.1% Tween 20

7. Conjugate
Affinity purified Horse Radish Peroxidase labeled anti-goat IgG (High and Low)

B) Test procedures

1. Indirect ELISA

Aim

Using an indirect ELISA, to assess whether goats develop antibodies against TSH administered to them under laboratory conditions.

Reagents

Purified bovine TSH

Rabbit anti-bovine TSH antibodies (serum) (positive control serum)

Goat pre-exposure serum to purified bovine TSH (negative control serum)

Goat serum post-exposure to purified bovine TSH.

IgG HRP-labeled anti-goat serum (a commercial reagent) diluted 1:2000

Wash buffer (Phosphate buffered saline (PBS)/ 0.1% tween 20)

2. Evaluation of the indirect technique controlling for the non specific effects of FSH and LH

Introduction

Both pre- and post-inoculation goat sera described above were used in the development of the ELISA for detection of levels of thyroid stimulating hormone in bovine sera.

Aim To evaluate an ELISA technique to detect TSH levels in cattle.

Materials

Pre-inoculation goat sera

Post inoculation goat sera

Thyroid stimulating hormone (TSH)

Follicle stimulating hormone (FSH)

Leutenising hormone (LH)

2,2'-azino-bis (3-ethyl-benzthiazolin 6-sulphonic acid) *alias* ABTS.

Phosphate buffered saline (PBS)

ELISA plates

Skimmed (non-fat))milk 3%

Horse radish peroxidase labeled anti-goat conjugate (KPL)

0.1% Tween 20

ELISA Reader with 405 nm lens.

Method

Indirect ELISA (using TSH, FSH and LH)

a) Coat plate with 100µl per well (2µg) of each of thyroid stimulating hormone, follicle stimulating hormone and leutenising hormone.

b) Incubate at 4^0 C overnight.

c) Wash plate 4 times in PBS/0.1% Tween 20.

d) Block with 200µl per well 3% skimmed milk and incubate for one hour at 37^0c.

e) Wash plate 4 times in PBS/0.1%. Tween 20.

f) Add 10µl per well pre and post inoculation goat sera diluted 1 in 1000 in 3% skimmed milk in PBS/0.1%. Tween 20 and incubate for one hour at 37^0c.

g) Wash plate 4 times in PBS/ 0.1% Tween 20.

h) Add 100µl per well Horse-radish peroxidase labeled anti-goat IgG (conjugate) diluted in 1:2000 in PBS 1% skimmed milk/ 0.1% Tween 20. Incubate at 37^0c for one hour.

i) Wash plate 4 times in PBS / Tween 20.

j) Add 100µl per well of ABTS substrate and incubate at room temperature for 20 minutes.

k) Read the optical density (OD) at 405nm.

The plate was coated with 100µl per well of each of TSH, FSH and LH. Pre- and post-inoculation goat sera were added to react with each hormone. Anti-goat conjugate was added followed by substrate and indicator. Optical densities were then read.

3. Removal of non-specific reaction by preadsorption of goat sera in TSH, FSH and LH

Pre and post inoculation goat sera were adsorbed in TSH, FSH and LH respectively by mixing equal volumes of the serum with each of the 3 hormones. The mixture was incubated at room temperature and then repeated at 37^0C. An indirect ELISA was performed using TSH.

Step 1

1. Coat plate with 100µl per well of hormones FSH, LH and TSH
2. a) Incubate overnight at 4^0C

   b) Wash 5 times with PBS
3. Block with 200µl blocking buffer (3% milk) for 1 hr at 37^0C
4. Dilute sera 1:100, 1:300, 1:900 in 3% milk
5. Add sera to plate at 100µl per well on each FSH, LH and TSH coated well
6. Incubate for 1 hr at 37^0C
7. Transfer 100µl of sera to test plate coated with TSH and carry out the indirect test.

Step 2

1. Coat plate with 100µl/well TSH (2µg) diluted in coating buffer
2. Incubate at 4^0C overnight
3. Wash plate 5 times with PBS
4. Block for 1 hour at 37^0C with 3% skimmed milk

5. Add 100µl/well of 1:100, 1:300, 1:900 pre and post sera adsorbed in FSH, LH and TSH
6. Incubate at 37^0C for 1 hour
7. Wash 5 times
8. Add 100µl per well conjugate diluted 1:200 (2-Goat – HRP)
9. Incubate 1hour at 37^0C
10. Wash 5 times with PBS
11. Add 100µl ABTS

Read OD at 405nm

4. Evaluating a sandwich ELISA (test for cross-reactivity) amongst TSH, LH and FSH

Aim:

To assess the level of cross reactivity of TSH, LH and FSH in an ELISA format.

Method

Bovine TSH (thyroid stimulating hormone) Sandwich ELISA

1. Coat plate with 100µl per well of goat anti-bovine TSH serum diluted 1 in 100 in coating buffer.
2. Incubate at 4^0c overnight
3. Wash plate 4 times in PBS/0.1% Tween 20.
4. Block plate with 200µl per well 3% skimmed milk in PBS/0.1% Tween 20.

5. Incubate at 37^0c for 1 hour.
6. Wash plate 4 times in PBS/0.1% Tween 20.
7. Add 100µl per well of 2-4µg of TSH}
   FSH} diluted in 3% skimmed milk
   LH } milk/PBS/0.1% Tween 20
8. Incubate at 37^0c for 1 hour.
9. Wash plate 4 times in PBS/0.1% Tween 20
10. Add 100µl per well of goat antibovine TSH serum (post inoculation goat serum) diluted in 100 in 1% skimmed milk/PBS/0.1% Tween 20.
11. Incubate at 37^0c for one hour.
12. Wash plate 4 times in PBS/0.1% Tween 20.
13. Add 100µl per well of Horse-radish peroxidase labeled anti-goat IgG diluted 1 in 2000 in 1% skimmed milk/PBS/0.1% Tween 20.
14. Incubate at 37^0c for one hour.
15. Wash plate 4 times in PBS/0.1% Tween 20.
16. Add 100µl per well ABTS substrate.
17. Incubate at room temperature on a plate shaker for 20 minutes.
18. Read optical density at 405nm

The plate was coated with goat antibovine TSH serum and reacted with thyroid stimulating hormone, Follicle stimulating hormone and leutinising hormone respectively in an attempt to minimise cross-reaction. TSH antigen was then added to the plates. After washing, pre and post inoculation goat sera were then added, followed by antigoat

conjugate. ABTS substrate was then added, and optical density read using a 405nm filter.

5. Checkerboard titrations of pre-inoculation goat sera, fetal calf sera and normal bovine sera (presumptively negative for TSH, FSH and LH) with anti-bovine TSH.

Aim

To optimise the sandwich ELISA test in view of the background interference in pre-exposure sera

Materials

Rabbit anti-bovine anti-serum

Normal laboratory bovine serum

Goat anti-bovine horse radish peroxidase labeled IgG

PBS with 0.1% Tween 20

ABTS substrate (KPL).

Method

Pre TSH exposure goat serum, normal bovine serum and fetal calf serum were titrated against different dilutions of anti-bovine TSH in a sandwich ELISA. The assumption being made was that the readings to be obtained from cattle sera could be more sensibly computed having evaluated the level of background reading in goat sera at defined optimal conditions.

APPENDIX II

DETERMINATION OF THE DOSAGE RATE OF POTASSIUM IODATE FOR CATTLE IN THE MAZOWE EXPERINTAL SUPPLEMENTATION TRIAL

Aim: to study the effect of supplementary iodine on the levels of FT_3 and FT_4 in serum of weaner cattle.

Materials

Potassium iodate (analytical grade) 83.6% pure (Milborrows Animal Health Pvt. Limited Harare).

Calculation

1) KIO_3 solvates in water as follows:

$KIO_{3\ (solid)} \longrightarrow$

$K^+(aq) + IO_3$ **(aq)**

In the biological system the IO_3 is further reduced to give iodine

In this reaction, 2 moles of KIO_3 are expected to yield 1 mole of iodine (I_2). The relative molecular weight if iodine is 253.8. Therefore one gram (mass) of pure iodine should contain 1/253.8 moles of iodine or 0.00394 moles, which are generated from 2 mole equivalents of KIO_3 that is 0.00788 moles of KIO_3 which has a relative molecular weight of 214.02.

These 0.00788 moles of KIO_3 are equivalent to 0.00788 * 214.02 grams of KIO_3 = 1,686 gram which therefore equates to 1 gram of iodine.

2) To make an aqueous solution containing 30mg iodine per 10ml:

a) dissolve 3000 mg in 1000 ml or 3 grams in 1 litre

b) Using KIO_3, this concentration is attained by dissolving 3 * 1.686 grams or 5.0594 grams of KIO_3 in water.

3) This concentration is adjusted for purity as follows:

100/83.6 (level of purity) * 5.0594 which gives 6.0519 grams KIO_3

Therefore 6.0519 grams of KIO_3 dissolved in water gives a solution for a dose rate of 30 mg per 10 ml.

4) The solution should be stored in a dark bottle at room temperature for stability.

APPENDIX III

MAP OF ZIMBABWE SHOWING GENERALISED CLASSIFICATION BY

NATURAL

AGRO-ECOLOGICAL REGIONS