HISTOPATHOLOGICAL AND IMMUNOHISTOLOGICAL STUDY OF CUTANEOUS TUMOURS AND OTHER SURFACE SWELLINGS OF HORSES IN KENYA

Dhaval Nileshkumar Shah

Supervisors:

Prof. P. K. Gathumbi (UoN)

Dr. J. K. Gathumbi (UoN)

Dr. V. S. Varma (UoN)

INTRODUCTION

- The total population of horses in Kenya is about 4,000 (**Jockey Club of Kenya, 2012**).
- Equine practice in Kenya has largely remained at the periphery, with most of the professionals focused in non-equine practice.
- Literature review indicates that there are no published reports in Kenya on incidence of abnormal surface growths and masses in horses.

INTRODUCTION - continued

- Surface lesions in the horse can be classified as inflammatory, neoplastic or miscellaneous.
- Inflammatory lesions may include hypersensitivity reactions (those resulting in urticaria, hives and eosinophilic granulomas), staphylococcal cellulitis, Pemphigus Foliaceus, and pyoderma (folliculitis).
- Neoplasms of the horse include: sarcoid, fibroma and fibrosarcoma, melanoma and melanosarcoma, squamous cell carcinoma, and lymphosarcoma.

INTRODUCTION - continued

- Miscellaneous surface lesions of the horse include seromas, haematomas, abscesses, papillomatosis, and chronic progressive lymphedema (**Crabbe and Carter, 2007; Gore *et al.*, 2008**).
- In the horse, most cutaneous masses and growths are tumorous (**Meuten, 2002**)
- Diagnosis of surface swellings in the horse mainly involves evaluation of clinical signs and histopathology on biopsies using standard histopathological methods and specific immunohistochemistry (**Crabbe and Carter, 2007; Gore *et al.*, 2008**).

PROPOPSED STUDY

- The proposed study will report the retrospective and prospective cases of tumorous and non-tumorous surface swellings in the horse in Kenya.
- The retrospective study will be based on histopathology and immunohistochemical evaluation of past cases submitted to the Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi since year 1967.
- The prospective study will evaluate recent cases since the year 2010

JUSTIFICATION

- At the moment there is no published documentation of tumorous and non-tumorous surface lesions of the horse in Kenya.
- Most of the diagnosis has been based on histopathology which is sometimes challenged in diagnosis of tumours that show close morphological similarities. In this case, immunohistochemistry with specific tumour markers can be used to confirm diagnosis of morphologically similar tumours.
- Some non-tumorous masses may occasionally be misdiagnosed as tumorous clinically. This justifies a histological and immunohistochemical confirmatory study.

OBJECTIVES

General Objective:

- To determine the pathology of the surface swellings of the horse in Kenya using a retrospective and prospective study of cases submitted to the Department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Nairobi.

Specific Objectives

- To determine the types of surface swellings using histopathological characterisation.
- To confirm the diagnosis of morphologically related tumours using immunohistochemistry.

LITERATURE REVIEW

- Although the equine skin consists of many layers and is tough and resilient, and generally heals quickly when injured, it is a common site of pathological masses and lesions.
- Some skin masses initially appear as minor lesions that can either remain as such, while others transform into major lesions if left unattended(**Crabbe and Carter, 2007; Gore *et al*., 2008**). The lesions range from inflammatory to tumour nodules.

LITERATURE REVIEW - continued

1. Inflammatory Skin Conditions

- **1.1 Hypersensitivity Reactions** - Hypersensitivity reactions in horses that produce cutaneous swellings include urticaria/hives and eosinophilic granulomas.

LITERATURE REVIEW - continued

Urticaria/Hives

- Urticaria is a reaction where the skin or mucous membranes swell because of fluid accumulation (oedema).
- These swellings are usually raised and small, but occur in large numbers.
- They often "pit" when on pressure.
- Hives are a common allergy problem in horses.
- These lumps might be triggered by reaction to a certain plant (ingested or contacted by the skin), fly bites, ingredient in a fly spray or shampoo, or reaction to the carrier in a vaccine.
- Hives can be an early indication of a more serious reaction.

LITERATURE REVIEW - continued

Eosinophilic granuloma

- This is usually a benign, raised, firm nodule above skin level that is often clinically confused with tumours.
- They are more common with *Habronema* larvae.
- Histopathology reveals the presence of granulomatous lesion with mixed inflammatory cells with dominant presence of eosinophils.

LITERATURE REVIEW - continued

- **1.2 Staphylococcal Cellulitis** - This is a severe *Staphylococcus* bacterial infection that spreads through tissue planes.
- These infections often occur in the limbs causing swelling, lameness, and skin damage.

LITERATURE REVIEW - continued

- **1.3 Pemphigus foliaceus** - This is an autoimmune disease that causes destruction of normal skin cells.
- The horse's immune system attacks normal skin tissue causing changes to skin cells.
- Initially, the affected areas are inflamed and develop fluid filled vesicles. These vesicles eventually rupture, leaving a break in the skin that begins to scale and crust.

LITERATURE REVIEW - continued

- **1.4 Pyoderma (folliculitis)** - This is inflammation of the hair follicles (due to fungal or bacterial infection) and is sometimes called pyoderma. It contains pus-filled pockets of bacterial skin infections (pustules).
- In horses, the pustules can be secondary to trauma or insect allergies.

LITERATURE REVIEW - continued

2. Neoplastic Skin Conditions

- Skin tumours are relatively common in horses of all breeds and ages.
- Tumours in horses tend to be locally invasive and slow to metastasize.
- There are three common types of skin tumours: sarcoids, melanomas, and squamous cell carcinomas.
- Many other different types of tumours can also occur but are not as common.

LITERATURE REVIEW - continued

- Early recognition, accurate diagnosis, and early treatment of skin tumours are crucial to obtaining relatively good success rates. Delayed recognition and treatment increase the chances of recurrence after treatment or metastasis (**Siegal, 1996; Hayes and Knightbridge, 2002; Meuten, 2002; Crabbe and Carter, 2007; Gore *et al.*, 2008; Jubb *et al.*, 2005; Wilson, 2011**).

LITERATURE REVIEW - continued

2.1 Sarcoid

- The cause of equine sarcoids is not exactly known. It has been suggested that sarcoids are the result of a combination of trauma, genetics, and an infection with a viral agent.
- Equine sarcoids are often classified as either verrucous (wart like), fibroblastic (proud flesh like), or as combinations of the two.
- Some sarcoids are small and inactive, with flat areas that are hairless, dry, rough, and grey in colour. Other sarcoids are raised, large, and active, often with the skin still intact.

LITERATURE REVIEW – continued

Sarcoid - continued

- A diagnosis can often be made based on the appearance of the lesions.
- If this is not possible, a biopsy or complete surgical removal can be performed.
- It is thought that some verrucous sarcoids change into more aggressive fibroblastic tumours when they are injured or traumatized. Because of this, partial biopsy of any verrucous tumour is not recommended.
- Histopathology :
  - Subepidermal proliferation of spindle-shaped to fusiform fibroblasts showing hyperchromasia with a moderate to high cell density, forming whorls, interlacing bundles and haphazard arrays with one another.
  - The mitotic rate is invariably low.
  - In the most typical cases, pseudoepitheliomatous hyperplasia and hyperkeratosis are observed.
  - There is marked formation of rete pegs, which are broad invaginations (up to more than 20 cells) of epidermal cells into the dermis.

LITERATURE REVIEW - continued

2.2 Fibroma and Fibrosarcoma

- These are mesenchymal tumours characterised by uncontrolled proliferation of fibrocytes or fibroblasts.
- There are two forms of fibromas: the soft fibroma (fibroma molle) consisting of many loosely connected cells and less fibrous tissue, and the hard fibroma (fibroma durum) consisting of a lot of fibrous tissue and few cells.

LITERATURE REVIEW - continued

2.3 Melanoma and Melanosarcoma

- They result from over-active melanoblasts (immature melanocytes).
- In horses a melanoma is thought to appear when abnormal melanin production occurs.
- Melanomas are firm, round, grey to dark, tumours that can occur singly or most commonly in multiple dark masses.
- These tumours can occur anywhere on the body being more common around the tail base, anus, and vulva. Melanomas are also seen on the limbs, penis, prepuce, udder, eyelids, and head.

LITERATURE REVIEW – continued

Melanoma/Melanosarcoma - continued

- Most melanocytic tumours are slow growing and do not metastasize. However, some tumours are fast growing, malignant, and can spread to the lymph nodes, liver, lungs, and spleen.
- If melanomas occur in coloured horses, they are much more likely to be malignant.
- A melanoma can be identified based on microscopic observations and by the presence of melanin pigment.
- **Valentine (2006)** reported that cutaneous and mucocutaneous melanomas are the second most commonly reported skin tumour of horses, accounting for up to 18% of all cutaneous neoplasms.

LITERATURE REVIEW – continued

2.4 Squamous Cell Carcinoma

- Squamous cell carcinoma is the most visible and readily detected skin tumour of horses.
- They may occur in sun-exposed areas such as the nares or eyelids, or in sun-exposed mucocutaneous junctions. It often occurs on the eyelid or around the vulva or sheath if the skin is unpigmented.
- It is the most common neoplasm of the equine eye and ocular adnexa and the second most common tumour of the horse overall (**Meuten, 2002**).

LITERATURE REVIEW – continued

Squamous Cell Carcinoma - continued

- **King *et al.* (1991)** reported that the most frequent site for ocular involvement of Squamous Cell Carcinoma is the nictitating membrane and conjunctiva. **Chahory *et al.* (2002)** reported that the cornea, the sclera, and the eyelids may also be involved with multiple and bilateral lesions occurring infrequently.
- According to **MacFadden and Pace (1991)**, Squamous Cell Carcinomas account for 20% of the cutaneous neoplasms seen in horses.
- Squamous cell carcinomas occur most commonly in adult to geriatric horses of any breed.
- They can be ulcerative or proliferative.

LITERATURE REVIEW – continued

2.5 Lymphosarcoma

- These are lymphoid tumours occurring as nodules all over the body in the generalised form.
- They can occur in the skin as raised nodules.
- Histopathologically, there is presence of immature forms of lymphocytes within the section forming nodules.
- **Rebhun and Piero (1997)** reported that Lymphosarcoma is the most common haematopoietic neoplasm forming cutaneous growths upon metastasis in horses.

LITERATURE REVIEW - continued

3. Miscellaneous Skin Conditions

3.1 Papillomatosis (Warts)

- Warts in horses are caused by the Equine Papilloma Virus.
- These lesions are harmless in almost all situations, except where pain is involved.
- Warts are spread by direct contact from animal to animal. They can also be spread when contaminated equipment (halters) is not cleaned properly between animals.
- **S**mall warts can be crushed, pinched off, frozen (cryosurgery), or surgically removed.

LITERATURE REVIEW – continued

- **3.2 Chronic Progressive Lymphedema**
- This is a condition that occurs in certain breeds such as Shires, Clydesdales and Belgians.
- The condition is characterized by swelling, thickening and scarring of the skin on the lower legs.
- The disease starts at an early age and progresses throughout the life of the horse, often resulting in disfigurement and disability of the limbs and sometimes premature death.
- The skin lesions on the lower limbs occur secondarily as a result of poor lymphatic drainage and tissue perfusion.
- The earliest lesions are characterised by skin thickening and crusting.

LITERATURE REVIEW – continued

- Secondary infections develop very easily, and both dark and white skin on the lower legs is equally affected.
- As the disease progresses, thick skin folds and sometimes multiple small, well-demarcated ulcerations develop predominantly in the rear of the pastern region.
- Severely affected horses often exhibit generalised swelling in all four legs. As the condition becomes more chronic, the lower leg enlargement becomes permanent and the swelling is firm to the touch.

LITERATURE REVIEW – continued

Immunohistochemistry

Immunohistochemical surface tumour markers include:

1. Cytokeratins

- These are intermediate filaments found in epithelial cells of all types and are specific markers for epithelial cell lineage.
- There are 20 subtypes which are organ- and tissue-specific. In addition, the subtype expressed depends on the stage in the sequence of terminal differentiation and the stage of development.
- An initial screen includes "pancytokeratin" characterized by the antibody AE1/AE3.
- Once the initial differentiation is made, more specific individual cytokeratins such as CK7 and CK20 can be used to better characterize an epithelial tumor.

LITERATURE REVIEW – continued
Immunohistochemistry - continued

2. Vimentin

- This is an intermediate filament that is present in most mesenchymal cells.
- It is found in almost all sarcomas and melanomas but is variable in lymphomas and even some carcinomas.
- It may be coexpressed with cytokine in a wide range of carcinoma and other tumors.

LITERATURE REVIEW – continued
Immunohistochemistry - continued

3. Melan A

- This is expressed by melanocytes and their neoplasms.

4. Proliferating Cell Nuclear Antigen (PCNA)

- This is expressed by proliferating cells.

MATERIALS AND METHODS

Study site

- The study will be conducted in close consultation with the equine practitioners in Kenya, especially those that are recognised by the Horse Association of Kenya and the Jockey Club of Kenya, in order to obtain the clinical background and the gross features of the surface masses in the horse.
- Histopathology and immunohistochemistry of the masses will be conducted at the Department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Nairobi.

MATERIALS AND METHODS - continued

Sample size determination

Sample size $$n = \frac{z^2 \cdot p \cdot q \cdot N}{e^2 (N-1) + z^2 \cdot p \cdot q}$$

Where: n = sample size

z = value of standard variate at confidence level

p = sample proportion (prevalence)

q = 1 – p

N = population size

e = confidence interval

MATERIALS AND METHODS – continued

Sample size determination - continued

- A pilot study had been undertaken and the prevalence was calculated to be 0.1 (p=0.1). The population size is estimated at 4000. The confidence interval is 0.05, thus z = 1.96
- Thus, the sample size will be:

$$n = \frac{1.96^2 * 0.1 * 0.9 * 4000}{0.05^2 (4000-1) + 1.96^2 * 0.1 * 0.9}$$

$$= 133.708148043302 5$$

$$\approx 134$$

MATERIALS AND METHODS - continued

Study design

Retrospective study

- The study will involve a retrospective data collection from archive records at the Department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Nairobi from January 1st 1967 to November 30th 2011.
- An initial study will be performed to identify relevant cases. The paraffin-wax embedded tissue blocks of the identified cases will be sectioned afresh using a microtome (two slides per block), stained with haematoxylin and eosin, and examined using a microscope.

MATERIALS AND METHODS – continued

Study design - continued

Prospective study

- Tissue specimens of cutaneous growths from horses will be processed, stained and examined histologically.
- A close liaison with equine practitioners in Kenya will be established to ensure that a thorough clinical record of the case and gross description of the sample will be made and that a sufficient number of samples will be collected. This will run from December 2012 to March 2013.

MATERIALS AND METHODS – continued

Study design - continued

Immunohistochemistry

- Immunohistochemical staining will be performed to confirm histological cases that will be difficult to diagnosis at histology.
- Reagents for immunohistochemistry will be obtained from Dako®, a global leader based in Denmark with over 40 years experience in the field of immunohistochemistry. The company has local representation in Kenya.

MATERIALS AND METHODS – continued

Study design - continued

- Briefly, the protocol for immunohistochemistry will involve application of standard protocols (**Bogaert *et al.*, 2010; Painter *et al.*, 2010**):
  - Deparaffinisation of the section.
  - Ceasing of endogenous peroxidase activity using 3% hydrogen peroxide-methanol.
  - Heat-induced epitope retrieval to enhance binding of the antibodies. This involves immersion of tissue sections in a pre-heated buffer solution and maintaining heat in a water bath (95–99 °C).
  - After thermal treatment, the jar with buffer and slides will be cooled for 20 minutes at room temperature and the slides rinsed well with buffer.
  - Incubation with primary antibodies, secondary antibodies and chromagen (Avidin-Biotin Complex detection system).
  - Counterstaining as applicable antigen.

MATERIALS AND METHODS – continued

Data collection

- Histopathology (H&E) – Each H&E stained slide will be examined.
- 50 randomly chosen fields will be examined at 40x objective to characterise the prominent histological changes. The frequency of the characteristic histological diagnostic features in these will be noted in order to confirm the diagnosis.

MATERIALS AND METHODS – continued

Data collection - continued

- Immunohistochemistry – The sections prepared according to standard protocols for immunohistochemistry and the manufacturer's guidelines will be examined for the antigen, its frequency, intensity and distribution. This will be done at 40x objective in 50 randomly chosen fields.
- The extent of histological lesion will be correlated with the occurrence, distribution and intensity of immunohistological features.

MATERIALS AND METHODS – continued

Analysis of data

- The data collected will be coded and input into Microsoft Office Excel 2007. Descriptive statistics will be derived. The results that will be generated will help in identifying the most commonly encountered diagnosis since 1967.

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WORK PLAN

Month	Aug 2012	Sep 2012	Oct 2012	Nov 2012	Dec 2012	Jan 2013	Feb 2013	Mar 2013	Apr 2013	May 2013	Jun 2013	Jul 2013
Proposal Writing	■	■	■	■								
Proposal Submission				■								
Data Collection				■	■	■	■	■				
Data Analysis								■	■			
Thesis Preparation									■	■	■	■
Thesis Submission												■

BUDGET

Description	Cost (KES)
Sample processing (600/= per sample – 134 x 2 samples minimum) includes prices for reagents and items required for standard histopathological techniques	160,800/=
Fuel for transport to and from the field (4,000/= per month – 5 months)	20,000/=
Stationery (Printing paper, printing ink, biro pens, etc.)	5,000/=
Immunohistochemistry kits (Cytokeratin, Keratin, Vimentin, PCNA)	100,000/=
Miscellaneous Equipment (Disposable Gloves, Surgical Blades, Disposable Masks)	10,000/=
Subtotal	**295,800/=**
Contingencies (10% of subtotal)	29,580/=
Total	**325,380/=**

The budget will be furnished by the investigator

Prices of imported items calculated from USD to KES at a rate of KES 85 per USD