THE EQUINE SARCOID: MOLECULAR AND EPIDEMIOLOGICAL STUDIES IN *EQUUS ASINUS*.

by

Stuart W.J. Reid B.V.M.S., M.R.C.V.S.

Thesis submitted for the Degree of Doctor of Philosophy
in the Faculty of Veterinary Medicine, University of Glasgow.

Department of Veterinary Medicine,
University of Glasgow
May 1992

ABSTRACT

The aim of this project was to investigate some aspects of the equine sarcoid of the donkey (*Equus asinus*). The study was undertaken in a large population of animals located at The Donkey Sanctuary, Sidford, Devon, in order to assess the feasibility of eventual vaccination against the disease. All material was derived from clinical cases and no animal experimentation was involved in the investigations.

The epidemiological studies were based upon information exported from the clinical records maintained on The Donkey Sanctuary mainframe computer. Of those donkeys developing sarcoids at The Sanctuary, the sex, age, age at which the animal entered The Sanctuary and the duration of stay were all shown to be factors affecting the likelihood of an individual donkey having sarcoids. In general, the disease was most likely to affect young male donkeys within the first four years at The Sanctuary and the tumours were most frequently observed in the paragenital region. A general linear model was constructed and it was demonstrated that male donkeys were, at best 1.1 times and, at worst, 4.5 times more likely to have sarcoids than females. The disease model was then used as a management tool for the identification of animals at the highest risk of developing sarcoids, in order to effect more prompt therapeutic intervention.

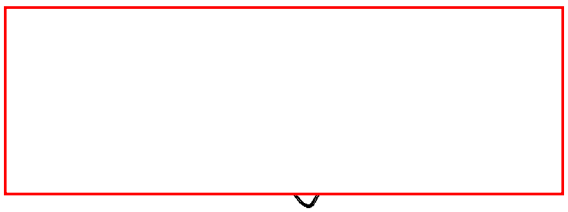
The serological status of donkeys at The Sanctuary was investigated by the immunoblot assay of sera from sarcoid-affected and clinically-normal donkeys for the presence of antibody to disrupted bovine papillomavirus type 2 (BPV-2) virion and to the L1 open reading frame (ORF) encoded fusion protein. Antibody to the antigenic targets was demonstrated in 86 per cent of donkeys in the case of the L1 fusion protein and 96 per cent of donkeys in the case of disrupted virion; there was no correlation between the presence of antibody and clinical status. The antibody detected by immunoblot was unable to neutralise the transforming activity of BPV-2 *in vitro*.

Attempts to isolate papillomavirus particles from sarcoids removed from donkeys were unsuccessful, although virions were demonstrated in an early sarcoid removed from a horse at the University of Glasgow Veterinary School.

The molecular investigations on 18 sarcoid tumours from The Sanctuary identified the presence of papillomaviral DNA homologous to BPV-2 DNA under stringent conditions, in all samples. Restriction endonuclease analysis of 15 of the tumours demonstrated papillomaviral DNA similar to BPV-1 and BPV-2. Nucleotide base sequence analysis of a cloned papillomaviral element from a sarcoid showed that the isolate was 95.6 and 98.0 per cent homologous to BPV-1 in the L1 and E5 ORFs, respectively. The results of the restriction endonuclease analysis were confirmed by the use of consensus primers and the polymerase chain reaction, localising an additional *Kpn*I site in four of the BPV-1-like samples to the region between the *Hin*dIII site and the origin of the viral genome.

DECLARATION

I declare that this thesis describes work carried out by me, except for those matters mentioned specifically in the acknowledgements. It has not been submitted in any form for another degree or professional qualification.



STUART W.J. REID

Parts of this thesis have been accepted for publication elsewhere. Chapter VII formed the basis for;

Reid, S.W.J. and Smith, K.T. (1992) The Equine sarcoid: Detection of papillomaviral DNA in sarcoid tumours by use of consensus primers and the polymerase chain reaction. *Equine Infectious Disease VI.* Proceedings of the Sixth International Conference on Equine Infectious Diseases, Cambridge, July 7th-11th, 1991. In press.

The literature review contains the basis for;

Reid S.W.J. and Howie, F. (1992) Factors associated with neoplastic disease in the horse. *Equine Veterinary Education,* **4**, 66-68.

ACKNOWLEDGEMENTS

Research, similar to most facets of life, is nothing if it is not a team effort; this project has been no exception. By virtue of the fact that several disciplines have been involved in the investigation of a disease at the molecular as well as at the population level, I have been fortunate to meet and work with many people. Without exception, they have given freely of their time and expertise, for which I am most grateful, and I apologise if I omit anyone from the following paragraphs.

First, without The Donkey Sanctuary there would have been no subject and it has been due to the efforts of Mrs. Elisabeth Svendsen, MBE, founder and administrator of the charity, that the importance of the sarcoid in the donkey population has been recognised. I am grateful to her for her advice and continued support throughout the period of research, and to the Trustees of the charity for granting the generous funds to finance the studies. All the staff at The Donkey Sanctuary deserve my thanks but especially the nurses, secretaries and the veterinary surgeons, who have made all trips to Devon enjoyable and productive. Special thanks go to John Fowler and Gill Whitehead and, more recently, Peter Ikin and Janet Eley whose efforts allowed me to do most of the work in Glasgow.

The initial period of the studentship was spent in the laboratories of Veterinary Pathology for which I have to thank Professors William Jarrett and David Onions. I also owe a debt to Professor Jarrett for his histopathological expertise, for advice in the adoption of PCR technology and continuing interest in the results of the project. I wish to record my appreciation to Drs. Lata Chandrachud and Jonathon Gaukroger for their instruction in serological assays, as well as Jackie Chalmers for guidance in the establishment of the immunoblot protocol. Electron microscopy was expertly performed by Dr. Helen Laird, Celia Burke and Marguerite Mason and the processing of histological sections by Ian McMillan, Jane Irvine and Lynn Stevenson, all of whom I thank. Messrs. James Murphy and Richard Irvine, also of Veterinary Pathology, gladly provided me with bovine skin samples and Graham Munroe of Veterinary Surgery, supplied sarcoids from his equine patients. Alan May's photography was of the highest order and I would like to thank Brian O'Neil, in particular, for his advice, friendship and guidance.

The molecular studies were initiated in the Cancer Research Campaign

me in already crowded conditions, without complaint, and I would like to thank Dr. Ken Smith especially, for his patient instruction. The PCR protocol was established under the supervision of Dr. Ruth Jarrett of the Leukaemia Virus Research Fund and the day to day bench work greatly eased by the generosity of Alice Gallagher. Richard Squires was also a source of advice, amusement and friendship, for which I am grateful.

And so to the department of Veterinary Medicine where the bulk of the project work was collated and where I will never be able to repay the favours I owe. Brian Wright was instrumental, sometimes, in the establishment of the facilities required for molecular biology in his laboratory. In this respect, the co-operation of the others in the lab., Arlene Macrae, Linda Forrest, June Downs and John Armstrong was essential and, more recently, Kirsty Maitland deserves great credit for continuing the work that was spawned by that contained in this thesis. Barbara Gillies and Cathie Love fielded telephone calls for me and and never complained when a fourth draft of a letter appeared for their attention. Dr. Mike Stear provided statistical advice and Dr. Chris Hunter criticized the PCR and serological protocols, usually constructively.

I owe a large debt to Professor George Gettinby for the time he spent explaining the rudiments of population statistics to me, usually in his own time and frequently at his family home. Carmel Mooney and Sean Callanan endured the trial of sharing an office with me during the writing-up period and my departmental colleagues, especially Dr. Sandy Love, went to great lengths to keep me in touch with matters clinical, for which I am grateful. The photograph of a type IV sarcoid on page ten was provided by Dr. Love.

Finally, my sincere thanks go to Professor Max Murray for his gentle criticism, forceful encouragement and friendly advice throughout the duration of the experimental work and, even more, during the production of this thesis. Colleagues and friends alike, it has been the establishment of the working relationships that has provided the most rewarding aspect of the project. Like all team games, it has been great fun.

This thesis is dedicated to my parents.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

A	adenine
AIDS	acquired immune deficiency syndrome
APS	ammonium persulphate
ASCII	American standard code for information interchange
BCG	Bacillus Calmette-Guerin
BCIP	bromochloroindolyl phosphate
bp	base-pair
BPV	bovine papillomavirus
C	cytosine
cm	centimetre
CMI	cell mediated immunity
CRPV	cottontail rabbit papillomavirus
D	density
DAB	diaminobenzidine
ddNTP	dideoxynucleotide triphosphate(e.g., ddCTP)
df	degrees of freedom
DMEM	Dulbecco's modification of Eagles medium
DMF	dmethyl formamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate (e.g., dATP, dTTP)
DoPV	donkey papillomavirus
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EEPV	European elk papillomavirus
EGF	epidermal growth factor
ELA	equine leucocyte antigen
ELISA	enzyme linked immunosorbant assay
FCS	fetal calf serum
g	gramme(s)
g	gravity
G	guanine
GLIM	General Linear Interactive Model
HPV	human papillomavirus
HRP	horseradish peroxidase
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobase

l	litre
LMP	low melting point
M	molar
MHC	major histocompatibilty complex
Mg^{2+}	ionised magnesium
ml	millilitre
mM	millimolar
MnPV	*Mastomys natalensis* papillomavirus
NBT	nitroblue tetrazolium
NCR	non coding region
nm	nanometre
OD	optical density
-OH	hydroxyl
OR	odds ratio
ORF	open reading frame
^{32}P	phosphorus-32
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PV	papillomavirus
RE	restriction endonuclease
RhPV	rhesus monkey papillomavirus
RI	refractive index
RNA	ribonucleic acid
RNAase	ribonuclease
rpm	revolutions per minute
RR	relative risk
^{35}S	sulphur-35
SDS	sodium dodecyl sulphate
ss	single-stranded
STD	sexually transmitted disease
T	thymine
TCTD	tissue culture transforming dose
TEMED	N,N,N',N'-tetramethyl ethylene diamine
U	unit of enzyme activity
ul	microlitre
uM	micromolar
URR	upstream regulatory region
UV	ultraviolet

CHAPTER I

GENERAL INTRODUCTION
AND
REVIEW OF THE LITERATURE

CHAPTER I

GENERAL INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. HISTORICAL BACKGROUND

The equine sarcoid was first recognised as a distinct tumour of the equine integument by Jackson in 1936. The term "sarcoid", adapted from human pathology and meaning sarcoma-like, was proposed in order to denote a specific disease entity, whilst allowing the pathologist to abdicate the responsibility of deciding whether the lesion was a true neoplasm or a chronic inflammatory process (Jackson, 1936). Although the definition has been widely accepted for over 55 years, various terms have been, and continue to be, used to describe sarcoids, including warts, angleberries, fibromas and dermal fibrosarcomas. There are many early references to warts in the literature. According to Fenner, Bachmann, Gibbs, Murphy, Studdert and White (1987), a stable master of the Caliph of Bagdad described equine warts in the ninth century and the clinical descriptions provided by Youatt (1866), Fitzwygram (1881) and Robertson (1883) are of lesions that would now be termed sarcoids.

1.2. NEOPLASTIC DISEASE IN THE HORSE

Sir Rupert Willis, cited by Cotran, Kumar and Robbins (1989), defined a neoplasm as "... an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change". The same authors (Cotran

equine neoplasia, stated that despite the extensive published literature, "tumours in horses do not in fact seem to be particularly common, either as medical or as surgical cases." However, the large number of papers which report or survey neoplastic disease in equidae (Cotchin cited over 100) may indicate that the prevalence of equine neoplasia is worthy of more extensive investigation.

In order to compare neoplastic disease in the different domestic species, adjustments which compensate for the vast interspecies life-span variation must be applied. Dorn and Priester (1987) applied Life-span Correction Factors based on maximum recorded life-span compared to man (Table 1). The results of this correction demonstrated that the estimated rate of neoplasia per 100,000 horses per year of life-span was approximately 2.5 times that of the rates for cattle, dogs and cats combined. In addition, the rate of malignant tumours in the horse was second only to that of the dog amongst the domestic species. However, correction for life-span does not take into account the influence of husbandry: Cats and dogs usually die as geriatric animals, horses may be allowed to survive their natural life-span but production animals rarely, if ever, survive beyond middle age.

Table 1. Estimated tumour rates of the domestic species, before and after correction for life-span. The correction factors are based on maximum recorded ages expressed as a proportion of an arbitrary 100 years for man (After Dorn and Priester, 1987).

		Estimated rate of tumours/100,000/Year					
	Adjustment	Unadjusted			Adjusted		
Species	factor	Benign	Malignant	Total	Benign	Malignant	Total
Cattle	0.30	49.7	177.2	226.9	14.9	53.2	68.1
Horses	0.46	2148.3	256.3	2404.6	988.2	117.9	1106.1
Cats	0.28	35.1	257.4	292.5	15.7	72.1	87.8
Dogs	0.20	643.7	828.3	1472.0	133.7	165.7	299.4

Neoplastic disease has been reported in every body system of the horse, for

(Schumacher, Smith and Morgan, 1988); Cardiovascular (Johnson, Beech and Saik, 1988); Lymphoid (Neufield, 1973); Ocular (Bistner, 1974); CNS (Bistner, Campbell, Shaw, Leininger, and Gholbrial, 1983); Urinary (Brown and Holt, 1985); Reproductive (Hinrichs and Hunt, 1990); Endocrine (Heinrichs, Baumgartner and Capen, 1990); Integumentary (Mullowney, 1985). However, the system most frequently affected is the integumentary system. In the survey of 12 North American Veterinary colleges, by Priester and Mantel (1971) and Priester (1973), skin tumours accounted for over 45 per cent of the total number of tumours. Ocular and genital lesions, the second and third most frequently affected systems, accounted for only 13 per cent and 10 per cent, respectively. This predominance of integumentary tumours may therefore bias any general conclusions about neoplastic disease in the horse.

Neoplastic disease includes both benign and malignant tumours, the latter more normally associated with the term "cancer". The perception of neoplasia in any species is undoubtedly influenced by the malignancy and/or life-threatening potential of the tumour, and any broad overview which classifies tumours by degree of malignancy may mask the fact that one tumour type in one system predominates and thus distorts interpretation of the data. Analysis of the equine data reported by Priester (1973), reveals that only 6 per cent of tumours of the integumentary system were malignant (the integumentary tumours together comprised over 45 per cent of all neoplasms) whilst 78 per cent of ocular and 52 per cent of genital tumours were malignant. It is evident from this that tumors of the skin and, in particular benign lesions, are the most commonly encountered manifestations of neoplastic disease in the horse.

1.3. TUMOURS OF THE EQUINE INTEGUMENT

With specific reference to the equine integument, Thomsett (1979) stated that there are few important skin tumours of the horse, listing the sarcoid, papilloma

range of tumour types including, the sarcoid, papilloma, squamous cell carcinoma, fibroma, melanoma, neurofibroma, keloid, lipoma, haemangioma, adenocarcinoma, lymphosarcoma, fibrosarcoma, sarcoma, adenoma, mast cell tumour, myxofibroma, sebaceous tumour, osteoma, sweat gland adenoma, adnexal carcinoma, basal cell tumour, carcinoma, haemangiopericytoma, histiocytoma, lymphangioma and a plasma cell tumour. The abundance of the benign lesions has already been mentioned (Priester, 1973) and the concensus of published surveys of tumour prevalence records the sarcoid as the most frequently diagnosed skin tumour by clinician and histopathologist. Two surveys from the United Kingdom conducted over 17 and 15.5 years report the sarcoid as the histopathological diagnosis in 60 per cent (Head, 1965) and 12.9 per cent of cases (Baker and Leyland, 1975), respectively. In two reports from the United States carried out over 10 and five year periods, the sarcoid accounted for 56 per cent (Strafuss, Smith, Dennis and Anthony, 1973) and 43.6 per cent of all equine tumours (Sundberg, Burnstein, Page, Kirkham and Robinson, 1977), respectively. A third report from North America, pertaining to the integument of the horse exclusively, recorded sarcoids as comprising 65 per cent of tumours over a two year period (Ragland, Keown and Gorham, 1970a). Australian workers also report sarcoids as the most commonly diagnosed skin tumour (Murray, Ladds and Campbell, 1978; Pascoe and Summers, 1981).

Mullowney (1985) stressed the difficulty in differentiating fibromas, fibrosarcomas and sarcoids and also commented that the majority of squamous cell carcinomas, papillomas and sarcoids were diagnosed clinically and could therefore be under-represented when analysing tumour occurrence, based on histopathological examination. With this in mind, Mullowney (1985) summarised ten reports of tumour occurence rates, including a total of 2005 tumours of the equine integument. The equine sarcoid accounted for 739 (36.8 per cent) of the total and was the single largest tumour group. Papillomata were the second most

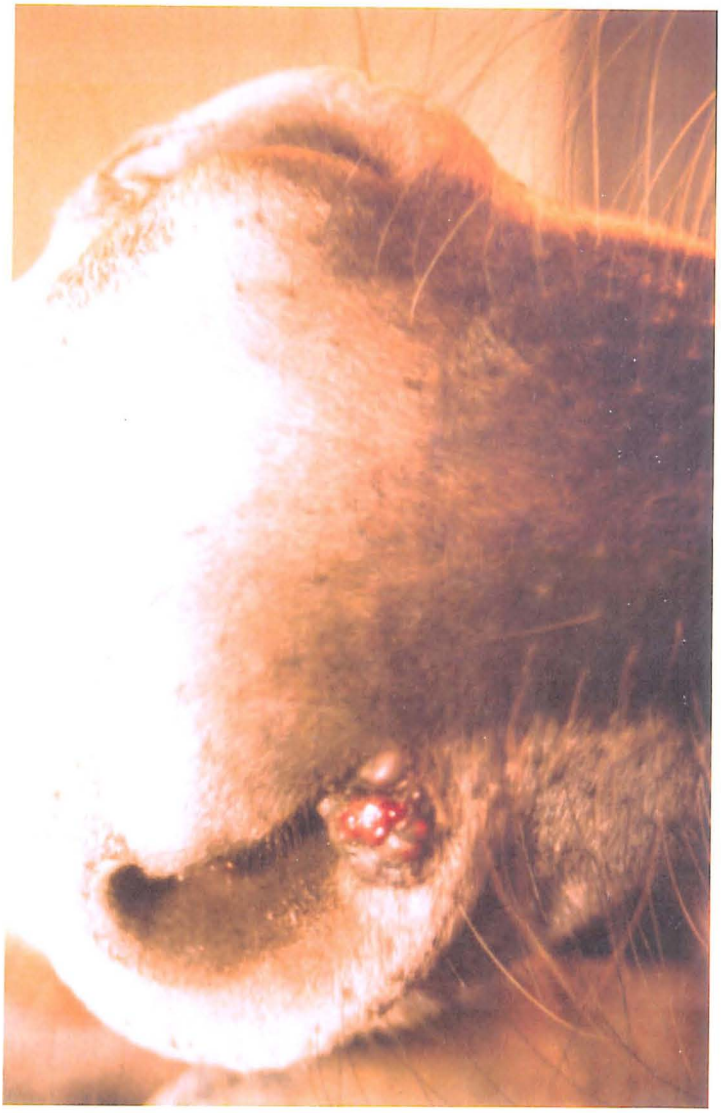
1.4. THE SARCOID

The sarcoid is a neoplasm affecting the skin of horses, donkeys and mules (Jackson, 1936; Olson, 1948; Ragland *et al.*, 1970a; Strafuss *et al.*, 1973; Genetzky, Biwer and Meyers, 1983)) and, although there is no reported sex or age predisposition (Ragland *et al.*, 1970a; Howarth, 1990), the tumour is generally observed in younger animals (Scott, 1988). The tumour is considered benign and non-metastatic but may infiltrate locally into the surrounding muscle tissue (Lane, 1977; Tarwid, Fretz and Clark, 1985), especially after surgical intervention (Pascoe and Summers, 1981). Sarcoids frequently occur on the site of previous insult to the skin (Lewis, 1964; Voss, 1969; Ragland *et al.*, 1970a) but may develop spontaneously (Olson, 1948) and it has been suggested that they may be spread either by direct contact or by fomites (Jackson, 1936; Olson, 1972). Animals may have multiple or solitary sarcoids with the head, ventral abdomen, paragenital region and lower limb being most frequently affected (Jackson, 1936; Ragland *et al.*, 1970a, Strafuss *et al.*, 1973; Murray *et al.*, 1978).

1.4.1. Clinical presentation

There are four clinical manifestations of the sarcoid described in the literature (Figures 1, 2, 3 and 4). Ragland *et al.* (1970a) classified sarcoids into three groups (types I, II and III) with a fourth category (type IV) added by Pascoe and Summers (1981). Type I or verrucose sarcoids resemble the cutaneous wart-like papillomas of bovine papillomavirus (BPV) infection of cattle; type II or fibroblastic sarcoids are fibrous nodules occurring within the dermis of the equine skin, although the fibroblastic component may sometimes ulcerate through the epidermis; type III sarcoids are a combination of types I and II and are termed mixed sarcoids with a fibroblastic component and verrucose epidermis; type IV or occult sarcoids are flat, slow-growing areas of alopecia containing small hard nodules within the dermis. Types I to III may be pedunculated or sessile (Ragland *et al.*, 1970a).

of the tumour (Ragland *et al.*, 1970a) and it was suggested by Jackson (1936) that the natural progression of the sarcoid development was from type I to type II, with eventual ulceration of the fibroblastic component through the epidermis. However, many sarcoids may remain quiescent for years with no increase in size and some sarcoids are first observed as type II lesions, suggesting that the sequence of events proposed by Jackson is not applicable to all cases of the disease (Ragland *et al.*, 1970a; Brown, 1983). Affected animals rarely reject sarcoids (Voss, 1969), the hosts immune system reacting minimally to the presence of the tumour (Gorman, 1985).



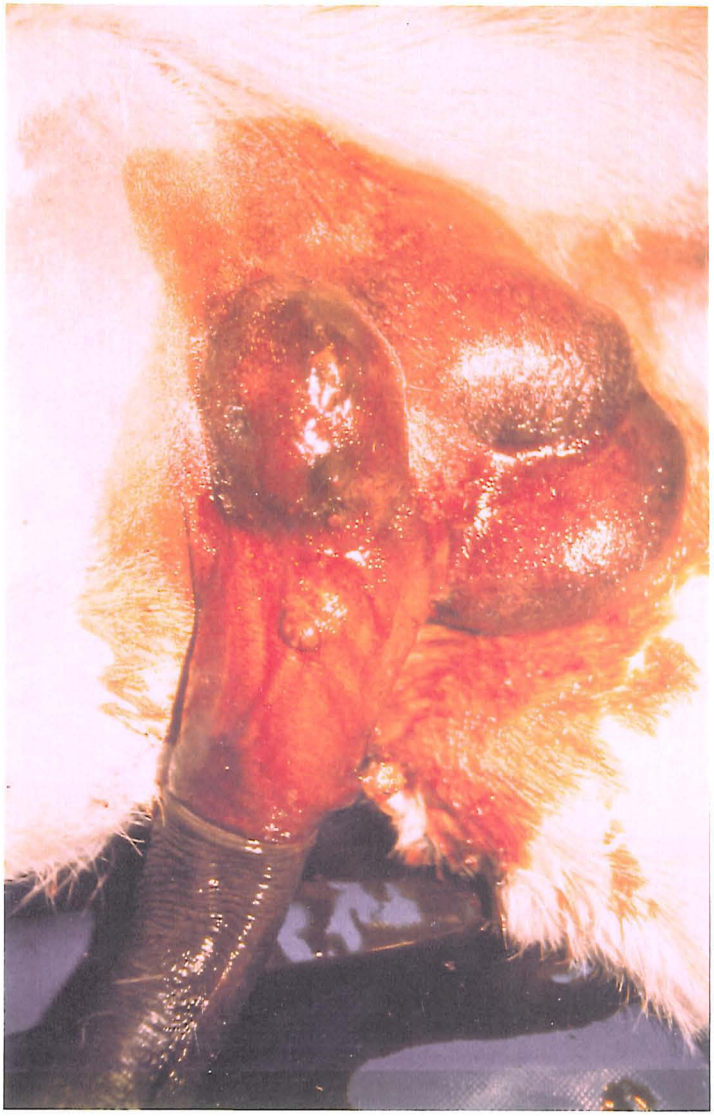


Figure 2. A type II or fibroblastic sarcoid located in the paragenital region of a male donkey.

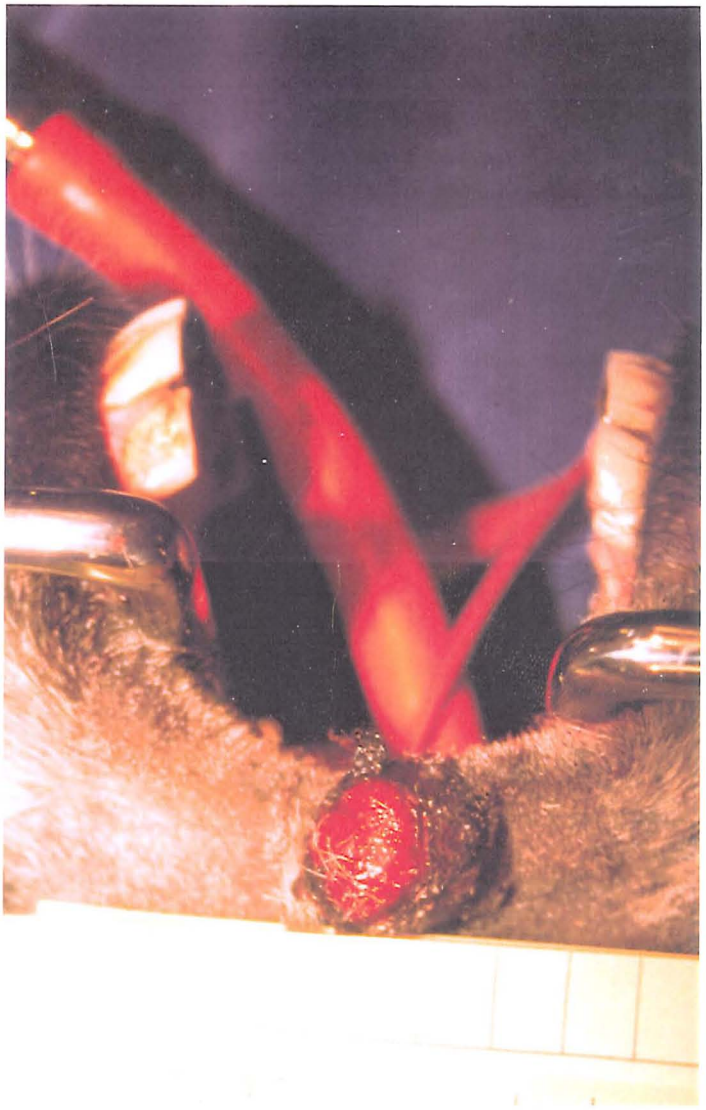


Figure 3. A type III or mixed sarcoid located at the commissure of the lips of a donkey. The lesion is ulcerated.

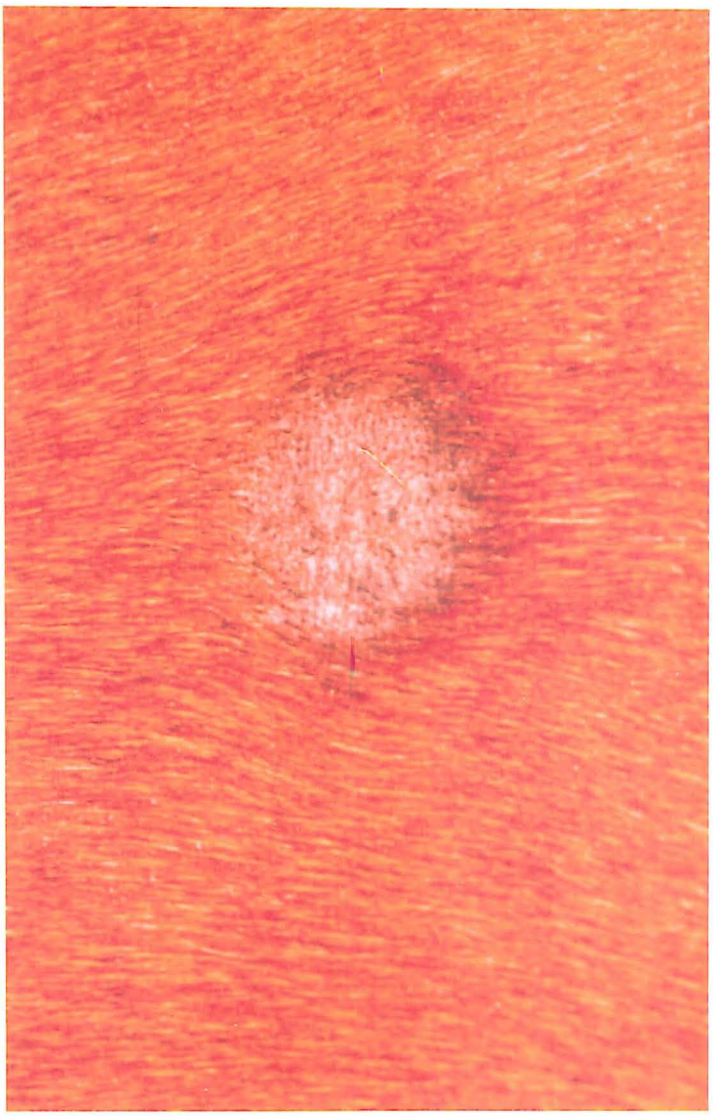


Figure 4. A type IV or occult sarcoid. There is an area of alopecia and slight nodularity of the skin surface.

1.4.2. Differential diagnosis

The equine sarcoid must be differentiated from all nodular and neoplastic conditions of the skin. In particular, the following diseases must be considered as major differentials (Pascoe and Summers, 1981; Brown, 1983; Tarwid *et al.,* 1985; Howarth, 1990):

Type I: Squamous papilloma, dermatophytosis, squamous cell carcinoma, cutaneous habronemiasis.

Type II: Nodular skin disease, fibroma, fibrosarcoma, neurofibroma, neurofibrosarcoma, melanoma, granulation tissue, cutaneous habronemiasis.

Type III : As for Types I and II.

Type IV:` Local inflammatory reactions, dermatophytosis, dermatophilosis, neurofibroma.

In many instances, although a tentative clinical diagnosis of sarcoid may be made, in order to obtain absolute confirmation, histological examination must be carried out (Howarth, 1990).

1.4.3. Histopathological features of the tumour

Histopathological changes that are reported vary depending upon the clinical type of the tumour and the degree of ulceration and trauma. Although Jackson (1936) originally suggested that the epidermis was an essential component for a positive diagnosis to be made, Ragland *et al.* (1970a) argued that dermal changes alone are sufficient for histopathological confirmation of a sarcoid.

The following features may be present in the epidermis: hyperkeratosis, parakeratosis, acanthosis, generalised epithelial hyperplasia and plexiform acanthosis (rete-peg formation) (Jackson, 1936; Ragland *et al.*, 1970a; Baker and Leyland, 1975; Pascoe and Summers, 1981; Tarwid *et al.,* 1985). Pathological features in the dermis include a lack of vascularity and proliferation of the dermal

1970a; Tarwid *et al.*, 1985; Murray *et al.*, 1978; Pascoe and Summers, 1981). In particular, the pallisading of fibroblasts and fibrocytes along the dermo-epidermal junction giving a picket-fence appearance, is associated with a sarcoid tumour (Ragland *et al.*, 1970a; Baker and Leyland, 1975; Tarwid *et al.*, 1985). The fibroblasts may be plump or spindle shaped and mitotic activity is variable. The composition of the dermis in the sarcoid differs from that of normal equine skin with type III collagen being more abundant (Williams, Heaton and McCullagh, 1982). This was attributed to the proliferative nature of the dermis in the tumour.

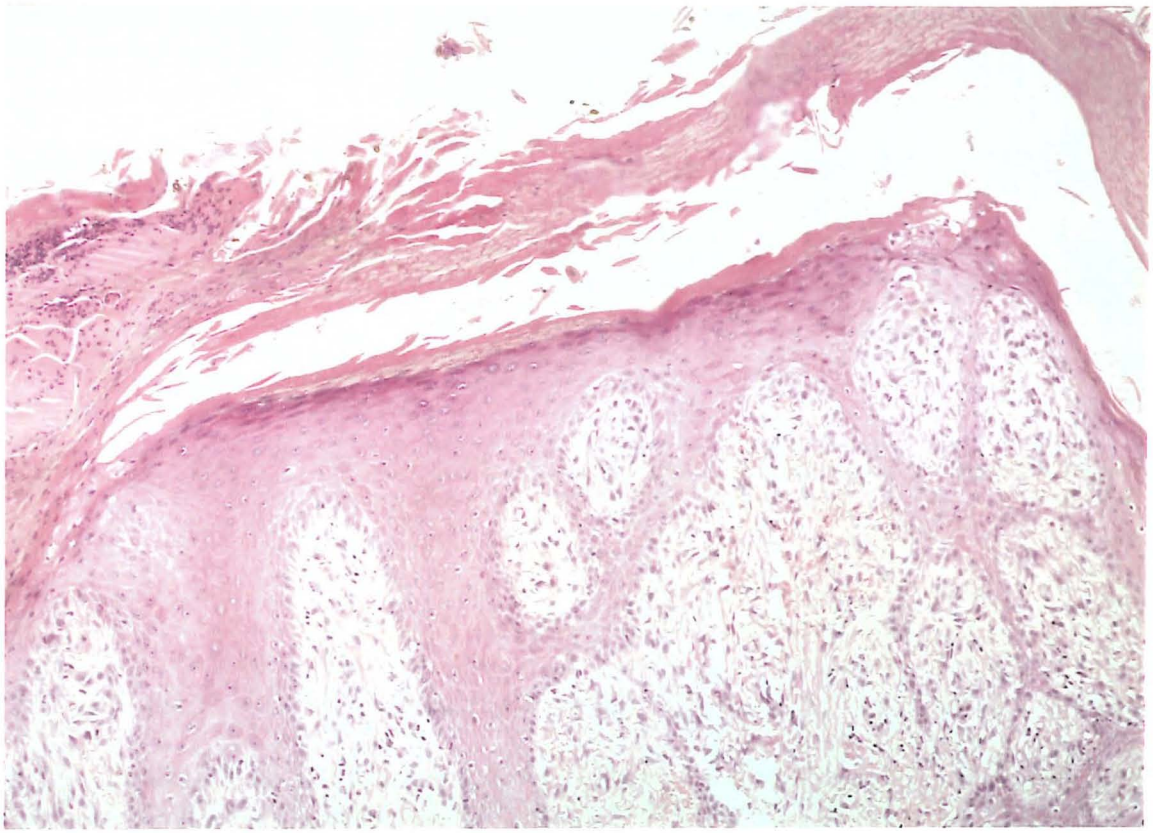


Figure 5. Histopathological appearance of a type I sarcoid showing characterisitics of a well developed fibropapilloma. There is hyperkeratosis, parakeratosis, acanthosis, retepeg formation

1.4.4. The genetic basis for the disease

The first suggestion that there may be a familial or genetic predisposition to the disease came from an epizootic of sarcoid reported by Ragland, Keown and Gorham in 1966 where four of five affected animals were members of the same family. This observation was supported by James (1968) who recorded the occurrence of sarcoids in three offspring out of a sarcoid-affected dam. James (1968) also suggested the possibility of vertical transmission of an aetiological agent.

Despite the fact that sarcoids are found in all breeds of horses, donkeys and mules, there are notable differences in the disease prevalence amongst different breeds. Angelos, Oppenheim, Rebhun, Mohammed, and Antczak (1988) found that quarter horses had nearly twice the risk of developing sarcoids as had thoroughbreds (Odds Ratio (OR) 1.8, $p<0.05$), whilst standardbreds had a much lower risk of developing sarcoids than other breeds (OR 0.2, $p<0.001$). More recently an association between susceptibility to sarcoids and the Major Histocompatibility Complex (MHC) has been demonstrated. In the horse, the MHC is known as the Equine Leucocyte Antigen (ELA) system and both class I and class II antigens have been reported at the population level in several breeds. Lazary, Gerber, Glatt and Straub (1985) recorded that the class I antigen W3 (A3) and the class II antigen W13 (B1) occurred more frequently in sarcoid affected horses whilst, in Irish horses with sarcoids, the W11 specificity was better represented compared with Swiss and French horses, which frequently were of W5 specificity. The association with W13 and A3 was confirmed by Meredith, Elser, Wolf, Soma, Donawick, and Lazary (1986) in the United States and by Brostrom, Fahlbrink, Dubath and Lazary (1988) in Swedish halfbred horses. Gerber, Dubath and Lazary (1988), in an analysis of multiple case families and half-sibling groups, showed that the W13 ELA haplotypes segregated with susceptibility to disease and, that in families not in possession of the W13 allele, other parental haplotypes may

1.4.5. Transmissibility of the sarcoid

Early experimental work on the equine sarcoid focused on the transmissibility of the tumour. Two groups of French workers first described the transmission of sarcoid-like tumours from one horse to another in the period prior to widespread acceptance of Jackson's term "sarcoid". Cadeac (1901) reported that extracts of a papillomatous tumour rubbed into the scarified skin of unaffected horses produced tumours in three to four weeks. Montpellier, Badens and Dieuzeide (1937) considered tumours observed on six animals to be of neural origin and later reported the autologous transplantation of one of the tumours on a mule (Montpellier, Dieuzeide and Badens, 1939).

Olson (1948) demonstrated the autotransplantation of sarcoid tumours into scarified equine skin but was unsuccessful in attempts to transfer the lesion by intradermal or subcutaneous inoculation of tumour suspension, or to demonstrate homotransplantation. Transmission of sarcoids to both affected (one of five) and unaffected (two of six) horses by inoculation of cell-free extracts was achieved by Voss (1969). However, sarcoid homogenates were also used in the same animals (Voss, 1969) and so the homologous transmission of the tumour by cell-free extract still required absolute confirmation. The incubation periods for the development of tumours in the experimental animals varied from 17 to 170 days in the case of autologous transplantations and from 50 to 184 days for transplantations to unaffected horses. Voss (1969) also recorded the early regression of both naturally-occurring and experimentally transferred sarcoids when autologous and homologous horses were infected with sarcoid tissue.

Ragland (1966) was largely unsuccessful in attempts to transmit sarcoids to nine unaffected horses, five unaffected donkeys and 12 wart-free calves by inoculation of cell-free extracts of sarcoid tumours. Most interestingly, two of the five donkeys developed growths, but Ragland, McLaughlin and Spencer (1970b) did not consider any of the 12 growths to be sarcoids. The tumours were aggressive

the fibroblasts and fibrocytes at the dermo-epidermal junction.

1.4.6. Aetiology

The original description of 44 cases of sarcoid by Jackson (1936) classified the lesions together with bovine and canine papillomas due to their gross clinical appearance and pathology. The canine wart was purely epithelial, unlike the bovine and equine lesions which possessed both epithelial and fibroblastic components. The association of the bovine papillomaviruses with the equine sarcoid was further supported by the work of Olson and Cook (1951) who inoculated bovine wart material intradermally in horses and produced nodules which appeared within 12 to 17 days. Some of these tumours resembled sarcoids histologically but most regressed spontaneously by 150 days, in contrast to naturally-occurring sarcoids which rarely undergo rejection (Voss, 1969). Cook and Olson (1951) were unable to induce tumours by inoculation on to scarified skin. Only one of the tumours induced in the study behaved biologically like a naturally-occurring sarcoid, persisting for over 18 months.

Ragland conducted a series of experiments in attempts to demonstrate BPV as the causal agent of the sarcoid (Ragland, 1966). The studies provided information on the ability of BPV to induce tumours in the equine integument, serological response to BPV inoculation and immunity to BPV infection in the horse. The results of these studies (Ragland and Spencer, 1969) showed that inoculation of BPV suspensions into young ponies produced lesions histologically similar to sarcoids but, unlike the natural disease, the tumours regressed within one year. In the same study, inoculation of BPV suspension into adult animals produced tumours in all of 21 animals but the lesions lacked the histological features of the naturally-occurring sarcoid and were rejected.

With regard to the serological reponse to BPV infection, Ragland and Spencer (1968) failed to demonstrate neutralising antibody to BPV in the sera

sarcoids. These animals also produced serum antibodies capable of neutralising BPV, unlike the animals with the natural infection. This work supported the findings of Segre, Olson and Hoerlein (1955) who demonstrated the presence of neutralising antibodies in the sera of horses with growths induced by the agent of bovine papillomas but contradicted the results published by Olson and Cook (1951) which reported no evidence of acquired immunity in animals inoculated intradermally with bovine wart extracts, as experimental horses were susceptible to repeated exposure. Ragland and Spencer (1968; 1969) and Ragland *et al.* (1970b) concluded that whilst BPV should still be considered a candidate for the aetiological agent of the equine sarcoid, the results of their studies did not provide definitive evidence that sarcoids were caused by BPV. The isolation of infectious papillomaviral particles has yet to be achieved (Gorman, 1985; Angelos, 1990) and it has been suggested that little advance will be made until a virus or viruses are isolated and characterised (Ragland *et al*, 1970b). Ragland and Spencer (1969) postulated that transmission attempts with viral nucleic acid might be an appropriate development of future research.

In the 1970s and 1980s, an alternative candidate emerged for consideration as aetiological agent following the establishment of a transformed cell line (Mc-1) from an equine sarcoid (Watson, England and Larson, 1972). A number of reports were published based on investigations involving the Mc-1 cell line which implicated a retrovirus in the pathogenesis of the tumour. The cells were shown to contain a C-type retrovirus (England, Watson and Larson, 1973; Cheevers, Roberson and Brassfield, 1982; Fatemi-Nainie, Anderson and Cheevers, 1982) and were tumorigenic in athymic nude mice (Fatemi-Nainie *et al.*, 1984) and in an Arabian foal with combined immunodeficiency disease (Cheevers, Roberson, Brassfield, Davis and Crawford, 1982). Animals with sarcoids were shown to have non-cytotoxic antibody to the Mc-1 cells (Watson and Larson, 1974) and a cell mediated immunity to Mc-1 cells by lymphocytes of sarcoid-affected animals was

Despite the fact that the retroviral element was subsequently shown to be present in normal equine skin cells and therefore presumably endogenous in origin (Cheevers, Fatemi-Nainie and Anderson, 1986) the possibilty that the virus is involved in the diseases process remains.

1.4.7. Molecular biology of the disease.

Investigations into the molecular biology of the sarcoid have supported the hypothesis that a BPV-like virus may be involved in the pathogenesis of the disease. The presence of papillomaviral DNA has been demonstrated in lesions removed from both horses and donkeys (Lancaster, Olson and Meinke, 1977; Lancaster, Theilen and Olson, 1979; Amtmann, Muller and Sauer, 1980; Lancaster, 1981; Trenfield, Spradbrow and Vanselow, 1985; Angelos, 1990; Angelos, Marti, Lazary and Carmichael, 1991). Initial studies were performed utilising DNA-DNA reassociation kinetics whilst more recent investigations, involving Southern blotting and hybridisation, have shown that the papillomaviral DNA does not integrate into the host genomic DNA, existing as non-integrated episomes with between 10 and 450 copies per cell (Amtmann *et al.*, 1980; Lancaster, 1981; Trenfield *et al.*,1985; Angelos *et al.,* 1991). The viral DNA may be present in a number of forms; supercoiled, open circle or linear are all demonstrable by Southern blotting and hybridisation to cloned BPV-1 or 2 DNA probes (Amtmann *et al.*, 1980; Lancaster, 1981; Angelos *et al.,* 1991). Restriction endonuclease digestion of the DNA extracted from the tumours has shown that a variety of restriction fragment patterns are present, some identical to BPV-1, some to BPV-2 and others similar to, but identical to neither BPV-1 nor 2. However, the high degree of homology demonstrated by the hybridisation studies performed at high stringency, suggest that the differences observed by Trenfield *et al.* (1985) and Angelos *et al.* (1991) are minor, perhaps differentiating viral subtypes. More recently, Teifke and Wiess (1991) utilised the polymerase chain reaction (PCR) to amplify a specific region of

and, by restriction enzyme digest with *Bst* XI, could detect no difference between the amplified DNA and the corresponding region of the BPV-1 genome.

1.4.8. Therapy of equine sarcoids

The therapeutic approach to the sarcoid has been diverse (Roberts, 1970; Genetzky *et al.,* 1983). Surgical excision, excision by diathermy, cryotherapy, excision by carbon dioxide laser, immunotherapy, hyperthermia, brachytherapy and chemotherapy have all been used alone or in combination and the relative merits of the various modalities have been reviewed (Roberts, 1970; Howarth, 1990). No one technique has been shown to be absolutely successful and the variation in responses to treatment may be due to the size, location or type of the lesion (Ragland *et al.* 1970a) and genotype of the animal (Sarcoid Workshop, 1990). No definitive comparison studies have been undertaken to elucidate the relationship between these factors and therapeutic outcome.

1.4.8.1. Surgical excision

Surgical excision using scalpel or diathermy may be performed under general or regional anaesthesia. Larger tumours or tumours occurring in less accessible anatomical locations usually require the animal to be under general anaethesia and the limiting factor in animals with multiple lesions is the duration of anaesthesia. Unfortunately, recurrence after tumour removal is frequent. Ragland *et al.* (1970a) reported a 50 per cent recurrence within three years of removal, whilst Diehl, Vingerhoets and Stornetta (1988) recorded a 64 per cent recurrence with many tumours regrowing before wound healing was completed. Other workers reported that the histological examination of tissue sections from the base of the tumour may provide guidance for the surgeon, ensuring complete removal of the neoplasm (Adams, Calderwood-Mays and Peyton, 1988). Split-thickness autogenous skin grafts have been advocated following radical excision of sarcoid

1.4.8.2. Cryotherapy

This modality of treatment has been used with success by several authors (Joyce, 1976; Lane, 1977; Fretz and Barber, 1980) and has the advantages of comparative safety to operator and patient and relative lack of scarring after healing. Several cryotherapeutic systems are available but liquid nitrogen is the cryogen of choice (Lane, 1977; Munroe, 1986). Cryotherapy is a destructive process killing both healthy and diseased tissue and, as a consequence, care must be taken when applying the cryogen which is best administered by spraying directly on to the affected area (Lane, 1977). The surrounding tissue should be protected from possible damage by applying petroleum jelly around the surgical site and an expanded polystyrene mask may be used to avoid liquid nitrogen run-off from the treatment area. Wicking materials such as gauze and towels should be avoided unless soaked in petroleum jelly (Krahwinkel, Merlkey and Howard, 1976). The use of thermocouple needles is advocated in order to monitor the temperature of the surrounding healthy tissue. The tumours should be frozen to a minimum temperature of -20^{o}C, although some authors report greater success following freezing to -30^{o}C (Fretz and Barber, 1980) or -50^{o}C (Rebhun, 1990) during two to three freeze/thaw cycles (Munroe, 1986). Debulking larger tumours may best be carried out by diathermy in order to keep the haemorrhage associated with sharp excision to a minimum, as excess blood at the freezing site leads to increased operating time during cryotherapy.

Sequelae to cryotherapy are, in general, predictable: Swelling local oedema and vasodilation, malodorous discharge often associated with superficial infection and eventual sloughing of the eschar occurs within two (Fretz and Barber, 1980) to eight weeks (Lane, 1977); cryodestruction of hair follicles and melanocytes may result in hair loss and depigmentation of the site. Untoward complications are associated with the accidental cryonecrosis of adjacent vital structures, including permanent and temporary facial nerve paralysis, irreversible damage to tendons,

Holmberg, 1980), lymphangitis and hind limb pain following the cryotherapy of a preputial tumour (Lane, 1977).

Generalised tumour regression of non-treated tumours has been reported following treatment of selected sarcoids (Lane, 1977) and may be due to an immune response following the release of altered cellular components. However, the spontaneous regression of tumours following cryotherapy is not a consistent finding.

1.4.8.3. Immunotherapy

Non-specific immunotherapy has recently found a place in the therapy of the sarcoid (Houlton, 1983; Schwartzmann, Cantrell, Ribi and Ward, 1984; Zeidner and Bracken, 1985; Rebhun, 1987). The Bacillus of Calmette and Guerin (BCG) is an attenuated strain of *Mycobacterium bovis*, an immunomodulator, and is the agent most commonly used for immunotherapy. Bast, Zbor and Boros (1974) noted a regression in tumours of the skin in patients following the administration of the BCG vaccine, the intralesional injection of the BCG inducing an immune reaction dependent upon the host's ability to mount a delayed (type IV) hypersensitivity response. In the therapy of the sarcoid, the organism has been use in a number of different forms: live (Wyman, Rings, Tarr and Alden, 1977), killed (Vanselow, Abetz and Jackson, 1988) or purified cell wall extracts (Murphy, Severin, Lavach, Hepler and Leuker, 1979; Lavach, Sullins, Roberts, Severin, Wheeler and Leuker, 1985) have all been combined or emulsified (Webster and Webster, 1985) with a variety of adjuvants and it is this diversity of preparation and protocol that makes the success of the technique difficult to assess. (Rebhun, 1990). Furthermore, the reponse to BCG administration appears to be related to the anatomical location of the sarcoid and to the clinical type - periocular sarcoids respond well (Wyman, *et al.*, 1977; Murphy *et al.*, 1979; Owen and Jaggar, 1987), whilst sarcoids on the limbs and in the axilla do not (Klein, 1986; Owen and Jagger,

tumour prior to immunotherapy.

The report of anaphylactic shock following second or third injections of BCG has led to the recommendation that animals should be premedicated with flunixen meglumine, corticosteroids and tripelenamine (Winston, Rings and Wyman, 1979; Owen and Jagger; 1987; Vanselow *et al.,* 1988). A number of therapeutic regimens have been described but all require repeated intralesional administration of the whole BCG organism or derivative at two to three week intervals on up to six occassions, injecting the BCG preparation at a rate of approximately $1ml/cm^2$ (Howarth, 1990). Response to therapy is more noticeable after the second or subsequent administration with local inflammation, developing to ulceration, necrosis and eventual rejection. Systemic reactions may include malaise, colic, diarrhoea, pyrexia, sweating, laminitis and transient leucocytosis (Howarth, 1990) with complete resolution of lesions taking up to 12 months. Hepler and Leuker (1980) considered the possibilty of anaphylaxis and aerolisation of BCG a hazard to both operator and patient.

Despite having been in use for over a decade, the precise mechanism by which the BCG exerts its action is still unclear. It would appear that for successful therapy the host must have a relatively small tumour burden and must be immunocompetent. An adequate dose of BCG must then be administered to the lesion and there must be close association of the BCG with the tumour cells. The persistence of the bacterial cell at the site of the tumour may be the stimulus for a chronic inflammatory reaction (Bast *et al.*, 1974) and so the choice of adjuvant may affect the success of therapy. The host immune system, which mounts little response to the sarcoid (Gorman, 1985), may be stimulated by BCG causing an increase in natural killer cells and activated macrophages (Bast *et al.*, 1974; Lavach *et al.*, 1985; Vanselow *et al.*, 1988). Vanselow *et al.* (1988) noted that the reponse appeared to be specific to tumour cells as the healthy tissue surrounding treated sarcoids was unaffected during tumour rejection, but Owen and Jagger (1987)

advantages of speed and apparent specificty, Howarth (1990) concluded that the cost of repeated visitations and premedication gave no significant economic advantage to BCG therapy over cryotherapy.

Other types of immunotherapy have been reported, in particular, the use of autogenous vaccines has been popular with field clinicians. Wheat (1964) proposed the use of an autologous vaccine, a suggestion supported by the favourable results of Page, Tiffany and Russell (1967) and by the early regression of naturally-occurring and transplanted sarcoids observed in autologous and homologous animals infected with whole sarcoid material by Voss (1969). However, Ragland *et al.,* (1970a) record the use of BPV vaccines as being of no use and Roberts (1970) obtained equivocal results using autogenous vaccines alone or in concert with adjuvants, cytotoxic drugs, antiviral drugs, BPV or other vaccines.

1.4.8.4. Chemotherapy

Cytostatic and cytotoxic drugs such as 5-fluorouracil (Bertone and McClure, 1990) and popophyllin (Metcalf, 1971) have been used to treat sarcoids in a number of topical applications, but Brown (1983) found their sole use unreliable. The technique was more successful when used in conjunction with other modalities.

1.4.8.5. Hyperthermia

This procedure has been reported to be successful in a number of cases (Hoffman, Kainer and Shideler (1983) but requires specialist equipment. Radiofrequency current (2MHz) is applied to a probe inserted in the tumour thus superheating the tumour cells and effecting cellular death. The technique may be synergistic with radiation therapy (Turrel, Stover and Gyorgyfalvy, 1983).

1.4.8.6. Interstitial Brachytherapy

A number of istopes have been used in the treatment of the sarcoid, implanting

for dedicated premises and the risk to operator and environment from misplaced or lost needles, excludes this technique from widespread usage.

1.4.8.7. Carbon dioxide laser

The most recent mode of therapy has been the use of carbon dioxide laser (Palmer, 1989). No recurrence after surgery was recorded 11 months after surgery. Diehl *et al.*, (1988) reported 81 per cent of 48 cases treated by this method had no recurrence of disease one year after surgery, comparing favourably with 64 per cent success in the 14 animals treated by cryotherapy after the same period of time. After carbon dioxide laser therapy the surgical site is dry with little or no haemorrhage. Primary closure may be attempted or the wound may be allowed to scab and slough leaving a bed of granulation tissue. The epithelialisation of the site may be slow, but the area is usually not painful on palpation (Diehl *et al.*, 1988).

1.4.8.8. Summary

It is evident that no one therapeutic approach is successful but Howarth (1990), although recommending cryosyrgery and BCG immunotherapy, comments that the long term approach to both treatment and prevention lies in the development of a specific equine sarcoid vaccine. Given the accumulating evidence implicating a papillomavirus in the disease, the following section contains a summary of salient information about papillomaviruses and the state of vaccine development against papillomavirus- induced disease.

1.5. THE PAPILLOMAVIRUSES.

1.5.1. Introduction

Papillomaviral infections are of clinical importance and may progress to malignancy (zur Hausen, 1985; 1991). The viruses induce a number of lesions in humans and animals and occupy an area of virology that is expanding rapidly,

cases per year) is greater than the incidence of leukaemia (11,000 new cases per year) in women and it is feared that in developing countries, which do not screen the female population, the rate may be five or six times that in the United States (Schreier, Allen, Laughlin and Gruber, 1988). To be of clinical significance papillomaviral associated lesions do not necessarily need to be malignant, as in the case of some of the genital lesions in humans (zur Hausen, 1991). Both the penile papillomas in cattle (Jarrett, 1985) and laryngeal papillomas in children (Steinberg and Abramson, 1985) are benign lesions but cause clinical problems to the afflicted and it is well documented that, on occasions these benign lesions may progress to malignancy as in the case of epidemodysplasia verruciformis. This is an inherited condition which manifests itself by the development of a number of papillomaviral induced lesions some of which may, after several years, become malignant (Orth, 1987).

The literature concerning the diseases caused by and associated with papillomaviruses is extensive and the reader is directed to several substantial reviews on both disease aspects as well as the biology and biochemistry of the viruses (Pfister, 1984; Howley and Schlegel, 1988; zur Hausen, 1991). The salient features with regard to the general properties are summarised in the following sections.

Until recently, the papillomaviruses have been grouped together with the polyomaviruses in the same family of Papovaviridiae. However, the two genera differ in biological behaviour as well as at the level of molecular organisation (Danos and Yaniv, 1983; Pfister, 1984). The polyomaviruses are usually non-oncogenic in their natural host, require integration of viral sequences into the cellular DNA for the transformed state to be maintained and both strands of the DNA viral genome code in the lytic cycle. By contrast, the papillomaviruses induce epithelial tumours in their natural host and the double-stranded viral genomes

Hausen, 1981; Favre, Jibard and Orth, 1982), as well as in transformed cells in culture (Groff and Lancaster, 1984; Law, Lowy, Dvoretsky and Howley, 1981; Moar *et al.* 1981a; Wood and Spradbrow, 1985). The DNA is found in supercoiled, open circle or linear forms (*vide supra*) and in addition, only one strand of the viral genome is transcribed (Amtmann and Sauer, 1982). As a result, the viruses are now classed as subfamilies of the Papovaviridae and the formal classification of the papillomaviruses is based on natural host and genome homology, with viruses demonstrating biological differences and less than 50 per cent homology being classed as different types.

The papillomavirus genome is broadly divided into three main domains; an early or transforming region, a late or capsid forming region and a non-coding region (NCR) also called the upstream regulatory region (URR), which contains elements involved in transcriptional control (Danos and Yaniv, 1983; Danos, Giri, Thierry and Yaniv, 1984). The early and late regions contain open reading frames (ORFs) which code for viral proteins and are responsible for the control of transformation, replication and viral assembly. The genomic organisation and function of the viruses will be discussed in more detail with specific reference to the BPVs.

1.5.2. Animal papillomaviruses

The first demonstration of the transmissibility of human warts by cell free extracts (Ciuffo, 1907) preceded, by more than two decades, the demonstration by Shope (1933) of the ability of the viruses to induce malignancy in rabbits (Shope, 1933). The viruses induced skin warts in cottontail rabbits in natural infections and in domestic rabbits under experimental conditions. Twenty-five per cent of the naturally-occurring and 75 per cent of the experimentally induced warts progressed to carcinomas (Rous and Beard, 1935; Syverton, 1952). In the experimental model, the papillomas were induced by intradermal inoculation of virion and malignant

caused neither papilloma nor carcinoma. Since then, papillomavirus infections have been reported in many animal species, e.g., goats (Davis and Kemper, 1936), horses (Cook and Olson, 1951), dogs (Cheville and Olson, 1964), monkeys (Koller and Olson, 1972), chaffinches (Osterhaus, Ellens and Horzinek, 1977), sheep (Gibbs, Smale and Lawman, 1975), mice (Muller and Gissman, 1978), hamsters (Graffi, Bender, Schramm, Kahn and Schneiders, 1969), deer (Shope, Mangold, McNamara and Dumbell, 1958), European elk (Moreno-Lopez, Pettersson, Dinter and Philipson, 1981) and cats (Carney, England, Hodgkin, Whiteley, Adkinson and Sundberg, 1990).

In order to understand the pathogenesis of papillomavirus infection the normal physiological development of the mammalian skin must first be considered. The epidermis in the mammal generally matures over a ten to 14 day interval with vertical differentiation of the basal cells. These cells mature first to the prickle cell layer (*stratum spinosum*) at which point the keratin gene expression commences (Franke, Moll, Achstaetter and Kuhn, 1986). This layer in turn matures into the granular layer (*stratum granulosum*) comprising between two and four rows of polygonal cells which lie parallel to the skin surface. The death of the epidermal cells begins at this stage as they become anucleate leaving the keratin precursor derived from the keratohyalin. The terminal layers consist of cells devoid of structure and these are continuously desquamated.

Research into the biology of papillomaviral infection has been hindered by the failure to propagate the virus *in vitro* and, as a consequence, the specific mechanisms and processes involved in the viral replicative cycle are poorly understood. The transcription, translation and viral assembly is intimately associated with the events of the maturing epidermis (Pfister, 1984). Infection of the skin by papillomaviruses requires the access to one or more basal cells (Jarrett, 1985) which proliferate leading to hyperplasia in the *stratum spinosum*. The virus can be demonstrated replicating in the *stratum spinosum* and *stratum granulosum*

layers. As the cell structures disintegrate, aggregates of viral particles can be demonstrated in the *stratum granulosum,* before finally being desquamated in the keratinised epithelium.

1.5.3. The bovine papillomaviruses

In recent years, there has been extensive research into the identification and characterisation of bovine papillomaviruses. To date, nine naturally-occurring bovine neoplasms have been associated with six characterised viruses (Campo, Moar, Laird and Jarrett, 1981; Jarrett, 1985) The bovine viruses are divided into two groups, A and B. Those viruses in subgroup A induce fibropapillomas with transformation of both epthelial cells and fibroblasts. Subgroup B viruses induce squamous epithelial neoplasms or "true" papillomas, the transformation being limited to the keratinocytes. The two groups can be distinguished immunologically as well as by the size and organisation of the viral genome and in both sub-groups the DNA is in episomal, non-integrated form. This extra chromosomal DNA occurs in multiple copies, usually between 10 and 200 copies per cell.

The viruses (Jarrett, 1985; Lancaster and Olson, 1982; Pfister, 1984) are typical of papillomaviruses having a 72 capsomere icosahedral capsid with a diameter of 55nm and containing a circular double stranded genome. The viruses belonging to subgroup A have many common attributes. BPV-1 has DNA sequence homology of 45 per cent to BPV-2 and 5 per cent to BPV-5. The molecular weight of the DNA is $5x10^6$ Daltons comprising approximately 7900 base pairs, the colinear genomes containing two major areas of conserved interspecific sequences in the E1 and L1 ORFs. These regions are also found in human and rabbit cutaneous papillomavirus. Agar Gel Immunodiffusion, Enzyme Linked Immmunsorbent Assay and Peroxidase Anti-Peroxidase demonstrate shared antigenic sites when reacted with rabbit sera to disrupted virus (BPV-1, 2 and 5) or by direct cross-reaction with bovine antisera from naturally infected

with the human, rabbit or subgroup A viruses. The genomes of the subgroup B viruses are smaller than those of BPV-1, 2 and 5 having a molecular weight of approximately 4.5 X 10^6 Daltons and a length of 7.2 kilobases. BPV-4 and BPV-6 share 50 per cent homology and BPV-4 and BPV-3, 20 per cent homology, under relaxed conditions. BPV-4 and BPV-3 do not possess the interspecific papillomavirus antigen.

The genome of the BPVs are typical of papillomaviruses having early, late and URR regions. The early region may contain up to eight ORFs, responsible for viral replication and transforming activities and which are expressed in fibroblasts, cultured cells and the basal cells of papillomas (Amtmann and Sauer, 1982; Pfister, 1984; Howley, 1986; Shah and Howley, 1990). The late region has two ORFs which code for the major structural proteins of the virus and these are only transcribed in the keratinising layers of papillomas and fibropapillomas, where the virus assembly takes place (Orth *et al.,* 1971; Amtmann and Sauer, 1982; Pfister, 1984)

Campo (1992) reviewed aspects of transformation of the animal papillomaviruses laying emphasis on the different means by which the viruses achieve transformation of cultured cells. The fibropapillomaviruses BPV-1 and 2 are able to transform primary cells without the need for additional oncogenes (Morgan and Meinke, 1980; Jarrett, 1985) and in this respect differ from the purely epitheliotropic viruses such as RhPV and BPV-4 which co-operation of the transforming gene with another oncogene, such as activated *ras* (Jaggar, Pennie, Smith, Jackson and Campo, 1990; Schneider, McGlennen, LaBresh, Ostrow and Faras, 1991). It is the early region of the papillomaviral genome which is necessary for transformation to take place, although the late region plays a significant role in some species such as CRPV (Meyers and Wettstein, 1991). In the case of BPV-1, it has been shown that the deletion of the entire late region results in no loss of transformation efficiency (Lowy, Dvoretzky, Shober, Law, Engel and Howley, 1980; Campo and Spandidos, 1983). The oncogenes responsible for transformation

E8 (Jaggar *et al.,* 1990; Jackson, Pennie, McCaffery, Smith, Grindlay and Campo, 1991); of CRPV are E6 and E7 with the E7 playing a minor role *in vitro* but an essential role in *vivo*. In the case of HPVs, HPV-16 requires E6 and E7, whilst the sole oncogene of HPV-8 is E6.

Of the several domains which have been associated with transformation (p105Rb binding region, casein kinase II phosphorylation sites and zinc binding cys-x-x-cys motifs) it is the zinc binding properties of BPV-1 E6 which are involved in cell transformation (Barbosa, Edmonds, Fisher, Schiller, Lowy and Vousden, 1990) and, in addition, the E6 is a putative transcriptional activator capable of disrupting gene transcription in the cell (Lamberti, Morrisey, Grossman and Androphy, 1990). The second major oncogene of BPV-1, E5, is responsible for the activation of EGF and PDGF (Martin, Vass, Schiller, Lowy and Velu, 1989; Petti, Nilson and Dimaio, 1991) and is also able to bind the 16K cellular protein component of the gap junction and vacuolar proton channel-forming ATPase, possibly resulting in the disruption of cell-cell communication and intracellular traffic respectively (Goldstein, Finbow, Andresson, McLean, Smith, Bubb and Shegel, 1991; Finbow, Pitts, Goldstein, Schlegel and Findlay, 1991).

1.5.4. Transmission of BPV

Whilst the exact method of transmission is still unkown, fibropapillomas in cattle appear to occur after a traumatic insult to the skin and a concommitant increase in mitotic activity. The appearance of tumours in cattle after winter housing may be related to areas excoriated by the animals on hay racks, posts or gates. Experimentally, calves may be infected with virus after making a deep scratch to the dermis, as reviewed by Jarrett (1985).

However anomolies exist. For instance, the lesions of the teats caused by BPV1, 5 and 6 are most commonly found in heifers and calves, suggesting that the previous *tenet,* associating milking machine trauma and calf suckling with

circulation of viral genomes. The source and route of infection of BPV-4 lesions in the alimentary tract is unknown.

Latency may also occur (Campo, 1987). Campo and Jarrett (1986) reported the occurrence of BPV-2 lesions on calves used in a BPV-4 transmission experiment. These calves had been removed from their dams at birth, reared in isolation in virgin accomodation and then infected wth BPV-4. BPV-2 lesions developed on sites where venipuncture had been performed and on traumatized skin of calves immunosuppressed by treatment with azathioprine. By Southern blotting and hybridisation, the lymphocytes of these animals were found to contain the BPV-2 genome, suggesting that latency in a genomic non-viral form may occur. These anomalies require resolution in order to elucidate fully the modes of transmission of the BPVs.

1.5.5. Vaccination against papillomaviral infections

Vaccination against viral disease is cited as being one of the goals of viral research (Schreier *et al.*, 1988). Although prevention of disease by prophylactic vaccination may be the ultimate goal, the related immunotherapies such as therapeutic vaccination have an equally important role to play, as their use may be more appropriate for ethical, medical, sociological, logistical or economic reasons.

For many years, inactivated tissue from naturally-occurring tumors has been used as a means of vaccination and this has been successful in some instances. Olson, Segre and Skidmore (1960) inoculated calves subcutaneously with formalinised, noninactivated bovine wart material or BPV propagated in chicken embryos. Although there was a variation in response, a good degree of immunity to intradermal challenge with BPV material was reported in the groups inoculated with formalinised or actively growing wart material, with multiple vaccinations providing better protection. The virus passaged in chick embryos was not effective

O'Neil, Gaukroger, Laird, Smith and Campo, 1990a) wherein calves vaccinated by deep intramuscular inoculation with homogenised tumour induced by BPV-2 or purified BPV-2 virus were both resistant to BPV-2 challenge. In contrast, the use of BPV-2 transformed fibroblast cultures as vaccines was unsuccessful and Jarrett *et al.* (1990a) concluded that the viral content alone was responsible for the protection against challenge. The same authors also suggested that the equivocal results obtained by workers in the field of human papillomavirus vaccination (Mallison, Morris and Jones, 1982) may have been due to to the heterogenity of the viruses involved in papillomaviral lesions, resulting in a vaccine being made from tumours induced by a different virus from the original pathogen. This possibilty was demonstrated effectively by the immunity of calves vaccinated with purified BPV-2, 4 or 6 to reinfection by homologous virus, and by the susceptibilty to challenge by BPV-1 of calves immune to BPV-6 (Jarrett, O'Neil, Gaukroger, Smith, Laird and Campo, 1990b). Furthermore, calves infected with BPV-2, 5 or 6 developed and rejected type specific lesions but were still susceptible to infection with BPV-4. The experiments indicated that prophylactic immunity to papillomavirus infection is type specific.

This research has progressed to the use of genetically engineered fusion proteins as vaccines (Jarrett, Smith, O'Neil, Gaukroger, Chandrachud, Grindlay, McGarvie and Campo, 1991). In these studies, the L1 and L2 encoded proteins of BPV-2 were expressed as beta-galactosidase fusion proteins and were used to vaccinate calves both prophylactically and therapeutically against BPV-2 infection. The L1 fusion protein was effective in preventing tumour formation when administered before challenge with BPV-2. The L1 was also effective in stimulating serum neutralising antibodies, probably accounting for the ability to confer prophylactic immunity. All animals vaccinated with the L1 fusion protein developed transient fibromas on challenge, findings in agreement with previous results published by Pilacinski, Glassman, Glassman, Reed, Lum, Marshall,

these studies also developed small fibromas but these decreased in size in the vaccinated animals. In Jarrett's study (1991), vaccination of calves with the BPV-2 L2 fusion protein was found to promote tumour rejection in vaccinated animals challenged with BPV-2, irrespective of whether the vaccine was administered pre- or post-challenge. This was accompanied by a massive infiltration of lymphocytes into the BPV-2 induced lesions, thus suggesting that the peptide used as a vaccine contained epitopes specific for T-cells involved in tumour rejection. Interestingly, Zhou, McIndoe, Davies, Sun and Crawford (1991) have shown that the expression of HPV-16 L1 in vaccinia virus can illict a priming of cytotoxic T lymphocytes in infected mice.

The use of molecularly cloned viral proteins as a means of immunotherapy has also been applied to those encoded by the early ORFs. Meneguzzi, Cerni, Kieny and Lathe (1991) immunised rats with vaccina viruses expressing the E6 and E7 proteins and were able to demonstrate the retardation or prevention of tumours induced by the inoculation of HPV-16-transformed cells.

1.6. SUMMARY

Research into vaccination against papillomaviral infections is advancing rapidly with the bovine model providing a system where direct experimentation is possible and, like the human disease, a multiplicity of viral types and clinical lesions have been characterised. Similarly, the equine sarcoid has several clinical manifestations and would appear to be non-permissive for viral replication like the cattle alimentary BPV lesions and human genital infections. Molecular investigations also suggest that different papillomaviral subtypes may be involved in the aetiopathogenesis of the disease. With this in mind, even though the use of bovine wart vaccines are considered to be of no (Ragland *et al.*, 1970a) or limited (Roberts, 1970) use, the sarcoid should still be considered as a disease suitable for either prophylactic or therapeutic vaccination.

in susceptible horses or donkeys. There have been no investigations into the transmission of the sarcoid, nor has the serological response in equids when challenged by specific papillomaviruses been researched. All of these subjects would require invasive, experimental techniques.

The Donkey Sanctuary is an animal welfare charity and was the grant awarding body of this research project. As a consequence, neither direct animal experimentation nor invasive techniques were employed in the investigative procedures employed during the course of the studies and, as such, challenge and transmission experiments are outwith the scope of this thesis. However, it is apparent that there is still a lack of knowledge regarding the epidemiology of the disease in large populations of horses and donkeys and it is also possible to investigate serological status *in vitro,* without the need to challenge animals, at least in the first instance. Furthermore, no aetiological agent has yet been satisfactorily isolated and characterised and there has been no attempt to correlate clinical lesions to the viral subtypes identified in molecular studies on the tumours. This work is central to an increased understanding of the disease and to any consideration of vaccination or immunotherapy.

The aim of the current project was the assessment of the feasibility of producing prophylactic or therapeutic vaccines to prevent or control the sarcoid, adopting a multidisciplinary approach to the study of epidemiological, serological and molecular aspects of the disease in a single large population of donkeys, with the ultimate objective being the improvement of animal welfare.

CHAPTER II

AN EPIDEMIOLOGICAL STUDY OF THE EQUINE SARCOID IN THE POPULATION OF DONKEYS AT THE DONKEY SANCTUARY

CHAPTER II

AN EPIDEMIOLOGICAL STUDY OF THE EQUINE SARCOID IN THE POPULATION OF DONKEYS AT THE DONKEY SANCTUARY

2.1. INTRODUCTION

Epidemiology may be defined as the study of a disease in a population and of the factors which influence its distribution (Lilienfeld, 1976). Numerous epidemiological surveys have been undertaken investigating neoplastic disease in humans. In a number of these studies, risk factors have been identified, e.g., urinary bladder neoplasia and industrial chemicals (Case, Hosker, McDonald and Pearson, 1954), but in the majority of cases there has been no direct causal relationship or aetiological agent demonstrated. Epidemiological studies of neoplastic disease in domestic animals have been few in number. Bendixen (1965) and Jarrett, Murphy, O'Neil and Laird (1978) demonstrated the value of large scale surveys leading to the postulation of the viral aetiology of enzootic bovine leukosis and the carcinogenic effects of the bracken fern, respectively.

To date, no aetiological agent has been definitively linked to the occurrence of neoplasia in any system of the horse or donkey. There are several examples of putative agents: the association of bovine papillomavirus with the sarcoid still lacks the experimental reproduction of lesions biologically identical to naturally occurring sarcoids; no retrovirus has been identified in connection with equine lymphosarcoma, in contrast to the disease in felidae (Jarrett, Crawford, Martin and Davie, 1964) and bovidae (Miller, Miller and Olson, 1972). Other factors, such as smegma (Thackray, 1978) and ultraviolet light (Hargis, 1981) have been reported

factors which may predispose animals to cancer.

2.1.1. Population

There are many variables involved in the monitoring and survey of disease. The first, and arguably most important, is the definition of the study population. Most of the published surveys and reviews are based on the referral populations of large specialist, research or teaching establishments and provide a sample of a diseased population (Priester and Mantel, 1971; Priester, 1973). The use of case-control studies, rare in the veterinary literature, may go some way to redressing the bias of this referral selection, but many of the common, easily treated neoplasms, encountered by clinicians in the field, are still excluded. In addition, without the use of census information, the true population size and consequently prevalence and incidence of any disease remain crude estimates. Dorn and Priester (1987) referred to the Report of the Surgeon General of the US Army (1939-1941) as providing the closest estimate of tumour rates in a normal working population. In the Fiscal year 1940, the rate of cancerous and benign tumours per 100,000 horses was 40 and 389, respectively, and of the population of horses that died or were destroyed, 11 and 6 per 100,000 respectively. This provides some indication of the predominance in the horse of non-life threatening benign neoplasms, such as the sarcoid.

2.1.2. Age

The incidence rate of tumours in man increases with increasing age (Ashley, 1990). The estimated relative risk of neoplasia in cats, dogs and cattle also increases as the animal ages (Priester, 1973). In the ageing horse, there is also an overall increase in estimated relative risk. However, closer analysis of the large scale survey of North American and Canadian University Veterinary Schools reveals that the rate for benign neoplasms of the skin in the horse actually decreases with

benign and malignant neoplasms in the dog which both increase with age (Priester, 1973). The decreasing rate in the horse was attributed to the large number of cases of papillomata in the younger animal.

2.1.3. Sex

With the exception of tumours derived from the primary or secondary sex organs, there are few reports of sex associated neoplasms in any of the domestic species. In human oncology, the occurrence of this class of tumour has generally been related to occupation, with males traditionally exposed to more environmental, especially industrial, carcinogens (Case *et al.,* 1954). With regard to equidae, the neoplasm with the strongest sex association is the squamous cell carcinoma (SCC). Moore and Kintner (1976) reported gastric SCC four times more frequently in males than in females; Junge, Sundberg and Lancaster (1984) and King, Priehs, Gum and Miller (1991) reported SCC male:female ratios of 2:1 and 1.8:1 respectively. Secondly, the adenoma of the pars intermedia of the pituitary is cited as being a disease of predominantly aged female animals (Barbet, Baxter and McMullan, 1991; Heinrichs, Baumgartner and Capen, 1990). However, there have been no large scale studies and, until proven otherwise, it would appear that, ahead of sex, the major risk factor for the Cushingoid syndrome associated with the neoplasm, is age.

2.1.4. Epidemiology of the equine sarcoid

The sarcoid is a neoplasm with no reported sex or age predisposition (Ragland *et al.* 1970a, Howarth 1990). Although Ragland *et al.* (1966) described an epizootic of the disease in a small group of horses and there have been several reports associating sarcoids with family, breed and leucocyte antigens (James, 1968; Lazary *et al.,* 1985; Meredith *et al.,* 1986; Brostrom *et al.,* 1988; Angelos *et al.,* 1988), there have been no data published on the epidemiology of the disease in the donkey nor

2.2. THE DONKEY SANCTUARY

The Donkey Sanctuary is a charitable organisation dedicated to the rescue and care of unwanted or neglected donkeys. The charity was founded by Elisabeth Svendsen in 1967 and has grown to be the fifth largest animal welfare charity and 59th largest charity of the 170,000 charities in the United Kingdom. The Sanctuary comprises of seven farms in Devon and Dorset in the South-west of England and is currently caring for over 3,000 donkeys.

2.2.1. Source of animals

Donkeys kept at The Sanctuary are derived from three sources:

(a) Donations: Donkeys are given to The Sanctuary when owners' circumstances change and are no longer compatible with donkey ownership. (b) Bequests: an animal may be left to The Sanctuary following the death of an owner. (c) Rescue cases: The Donkey Sanctuary supports a national inspectorate which deals with cases of cruelty and neglect, either reported directly to The Sanctuary or by liaison with other animal welfare charities.

2.2.2. Admission of animals

On admission to The Donkey Sanctuary donkeys are housed in the Isolation Unit situated close to the centre of the main farm but 800 metres from other donkeys at Slade House Farm, which houses the main administration block and the veterinary hospital. The maximum size of an isolation group is ten animals and the maximum time span from the entry of the first donkey to the entry of the tenth donkey is three weeks. The donkeys remain in Isolation for a minimum period of six weeks during which time they are monitored for signs of disease or stress. Donkey stallions are isolated individually and are castrated before release from Isolation.

The animals are weighed weekly whilst in Isolation and are blood sampled weekly in order to monitor haematological and plasma biochemical parameters.

2.2.3. Routine Husbandry

During the isolation period the donkeys are subjected to two physical examinations. The first is performed within three days of admission to The Sanctuary and is carried out by two veterinary nurses. The donkeys are examined for obvious signs of disease and a record taken of the physical characteristics of the animal for identification purposes, including age, sex, colour, size and weight, the age being estimated by inspection of the animals incisors. The second examination is performed by one of the two veterinary surgeons permanently employed by The Sanctuary and takes place within 30 days of admission to the Isolation Unit. In addition to confirming the findings of the nurses, this detailed clinical examination identifies animals with previous or current disease and conformational abnormalities. The veterinary surgeon is responsible for the assignment of the donkey to a management group on release from Isolation. These groups are designed with the requirements of individual donkeys within a herd management structure and include: *Blind Group*: These donkeys are diagnosed as having a deficit in visual function and are kept together on level ground with no obstacles; *Peat Group*: Donkeys with clinical or historical evidence of respiratory disease, especially chronic obstructive pulmonary disease, are bedded on peat and fed pre-soaked hay; *Geriatric group; Obese group; Thin Group; Mare and Foal Group; Sarcoid Group.*

After admission, the animals are cared for by farm managers receiving veterinary attention only when necessary. The donkeys are weighed monthly and approximately 10 per cent of the animals on any one farm are randomly selected for blood sampling in order to monitor haematological and biochemical parameters. Any animal developing a sarcoid lesion is removed to the veterinary hospital for therapy and is usually re-allocated to the Sarcoid Group. Throughout the remainder of the animal's life a computerised record is kept of all diseases, veterinary treatments or change of location. Healthy, good natured animals that

"Rehabs" but remain under the care of The Sanctuary and are regularly visited by Sanctuary inpectors.

Donkeys form very close social bonds to one or more donkeys (Svendsen, 1989). These donkey "friends" may be relation or non-relation and, whenever possible such animals are kept together, at The Sanctuary, at Rehab or within the veterinary hospital.

2.3. MATERIALS AND METHODS

2.3.1. Definition of study populations

The study population was assembled from the computerised health records of all the animals admitted to The Donkey Sanctuary between 1967 and July 1990. A "Case" was defined as an animal that was observed to have a sarcoid 6 months or more after entering The Sanctuary. The exclusion period of 6 months was used in order to ensure that the likelihood of the disease having been contracted before entering The Sanctuary was kept to minimum, based on the observations of Voss (1969). Any animal presenting with a sarcoid at entry, during the Isolation period or within the first six months at The Sanctuary was allocated to a Pre-entry case group. The remainder of the animals were assigned to the Control group.

The three groups of animals identified above, i.e., Controls, Case, and Pre-entries, contained data from animals inelligible for inclusion in the study:

a) Direct Relinquishments: These animals were transferred directly from one foster home to another and had consequently never physically entered The Sanctuary.

b) Rehabs and returns: The animals in this group were currently, or had been, at foster homes and had therefore been exposed to factors outside The Sanctuary. There were no reliable or accurate animal histories for the period during which they were at not at The Sanctuary.

c) French Donkeys: Donkeys in this group were located in France and had never

and were subject to different husbandry.

These exclusions were applied to all three groups. In addition, animals in the first six months of their stay at The Donkey Sanctuary were excluded from the Control population because at that time it was not known whether the animals may develop a sarcoid and so be assigned to the Pre-entry population. In this manner three populations were identified:

I Cases

II Pre-entries

III Controls

2.3.2. Manipulation of electronic data and databases

Having defined the study populations, access to the clinical details of the individual animals at The Sanctuary was required in order to marshal the data in a format suitable for interrogation. A database is a structured collection of data stored in a consistent fashion within records which have a common format. (Gettinby, Thorpe and Anderson, 1989) and the database affords several important facilities central to the rapid organisation and interrogation of large data sets. Of particular importance is the Query command which may be used to access information suitable for statistical analysis. For example, [SEX]=1 and [AGE]=0.000..0.999 would be entered to identify all males (geldings) under 1 year of age. In order to create databases for each of the three populations described above, i.e., Case, Pre-entry and Control populations, access to the information stored on The Donkey Sanctuary mainframe computer was necessary. Unfortunately, the system in place at The Donkey Sanctuary had been installed with the administrative tasks of the charity given priority and none of the information relevant to animal health and welfare was in a format suitable for easy access or interrogation. In addition, the existing system was not compatible with IBM machines and was administered by a private company of computer consultants, who were responsible for both hardware

Sanctuary's business and without recourse to the computer consultants, all animal details should be transferred in a suitable format for manipulation on IBM compatible computers, which would allow data analysis to take place in Glasgow.

In order to achieve this, the clinical records at The Donkey Sanctuary were transferred to eight 1.4 megabyte diskettes in ASCII format using a customised Pack/Unpack archiving programme. Two discrete files were created and exported from The Donkey Sanctuary mainframe. The first was a complete record of all the clinical histories of the donkeys at The Sanctuary and was used solely to identify those animals which had been diagnosed as having a sarcoid. This was performed by importing the data on the diskettes into a proprietary wordprocessing package (SmartWareII, Informix Software Inc.) where they were managed as individual text files. Each record was then screened for specific strings of characters using the GOTO command, locating the specified string, regardless of case. The following strings were located: [?sarc?], [?tumour?], [?lump?], [?neop?], [?wart?], [?lip?], [?oma?], [?fibro?], [?papill?], [?mass?]. The "?" indicated a wild character or characters. On completion of the screening, each case containing one or more of the strings was committed to hard copy for inspection and confirmation by the clinician involved in making the original diagnosis, in the light of any subsequent examinations or histological reports. In this manner all the cases were designated either "Sarcoid" or "Non-sarcoid".

The second ASCII file exported from The Donkey Sanctuary mainframe was a complete record of all the animals at The Donkey Sanctuary, and this set of data contained the animal's name; number; sex; location; date of birth; date of entry; date of death, if applicable; age at July 1990; a list of close friends and relatives. The information was sent via the Wordprocessor and Spreadsheet modules to the Data manager module and a database constructed containing each detail listed above as an individual field within the database. Calculated fields containing age at entry and length of time at the Sanctuary were added. The close

animal fitted the definition of Case, Pre-entry or Control as outlined previously. The Case database, in addition to containing the details described above (i.e., the animal's name; number; sex; location; date of birth; date of entry to The Sanctuary; date of death if applicable; the age at July 1990) also contained the date the sarcoid was first observed. From these data fields the age at entry, the age at which the animal was first observed to have a lesion and the length of time spent at The Sanctuary until diagnosis of a sarcoid, were calculated. Dead Control donkeys were classified according to the age at which they died.

Thus, three databases were created, one each for Control, Case and Pre-entry population and the databases were then interrogated extracting data under the headings: sex; age at entry; age; age at observation of lesion; length of time at The Sanctuary (exposure); age at death. The summary data generated by the interrogation was then arranged in a fromat suitable for statistical analysis and the three populations compared and any differences tested for significance.

The possibility of disease transmission within the Isolation Unit was investigated by comparing the ratios of Cases and Controls that may have occupied the Isolation Unit at the same time as another Case animal, a Pre-entry animal or both. This investigation of the risk of being in contact with a sarcoid-affected donkey was also extended to those in close contact once outside the Isolation Unit by comparing the prevalence of animals defined as close friends and relatives with sarcoids to that of the general population.

2.3.3. Software and data analysis

All manipulation of electronic data was performed using SmartWareII, Informix Software Inc.. This package was considered most suitable because it comprised integrated modules and allowed for the transmission of data from one module to another with comparative ease. Statistical analysis of the data was carried out on MINITAB release 7.1 (Minitab Inc.) and the statistical modelling, employing the

Ltd and the Royal Statistical Society). All statistical tests were carried using the 5% significance level.

2.3.4. Disease Model.

In order to investigate the significance of the different variables outlined by the summary analyses, it was necessary to employ linear regression analysis. The use of a General Linear Model allows the combination of a number of factors in order to create a disease model and it is then possible to assess the risk of disease to an individual donkey given the specific animal details required by the model as well as assessing the effect of the individual risk factors on the likelihood of disease. The variables used in the present study were sex, age at entry and exposure, all of which are independent. The age at lesion data were not used in the modelling process as this variable may be affected by age at entry and may affect age at lesion i.e., old animals entering The Sanctuary will be older animals when they get the disease; older animals may have been exposed for longer.

The GLIM package is a tool for fitting generalised linear models to data, in which the modelling process is where the data are matched by a set of theoretical values. These theoretical values are derived from a small number of basic quantities, or parameters, and are close to the original data. The process requires two decisions: first, the choice of the relationship between the theoretical values and the underlying parameters; second, the choice of a measure of discrepancy which defines how close the theoretical values are to the data. The first of these choices relates to the systematic component of the model and has a linear predictor, which is a combination of explanatory variables with a set of parameters, wherein the predictor is related to the theoretical value by the link function. The second choice relates to the random component and allows the data to have a specified distribution. In the current project, two types of data were explored, one based on counts of diseased animals in which the selected distribution was Poisson

defined the models, the GLIM package then fitted the model choosing estimates of the parameters with values which minimised the deviance or discrepancy.

Thus, data concerning three risk variables, viz., sex, age at entry and exposure, were entered into a GLIM software program in order to constuct two disease models. First, in a log linear regression, the absolute numbers of animals in the Case population were entered and the effect of the interaction of the three variables deducted. This provided a basic maximal model, which was assessed for goodness of fit to the data by the scaled deviance and degrees of freedom on the assumption that the scaled deviance was distributed approximately as X^2. If the scaled deviance had a value insignificant at given degrees of freedom then the maximal model was considered to fit the observed data. Each two-way interaction was removed from the model and the significance of the effect of each two-way interaction assessed by the ratio of the change in the scaled deviance to the change in the degrees of freedom, again by use of X^2 distribution. The model which fitted the data with the inclusion of fewest terms was considered the minimal model. This process was then repeated entering the proportion of the population affected for any given variable in a logit model.

The GLIM software was then used to generate a table of the estimated risks of sarcoid in specified groups of donkeys, based upon the fact that for small values of p (i.e. small risks) the odds ratio (OR) is a close approximation to the ratio of the actual risks (relative risk, RR) and so it is possible to use the calculated actual risks to assess which animals are most at risk from the disease. Given that the odds of an animal having the disease is equal to the ratio of the chance of having the disease (p) to the chance of not having the disease (1-p), the log-odds for the outcome is *log*(p/1-p). The parameter estimates then represent the average changes on this scale expected for unit changes in the model variables. For two different animals, differing in sex only, the difference in the log odds is due to the difference on the logistic scale of being either male (*m*) or female (*f*) i.e.,

become:

$$log(p_m/(1-p_m)) = a \text{ and } log\ (p_f/(1-p_f)) = a+b,$$

therefore, $$log((p_m/(1-p_m))((1-p)_f/p_f)) = b$$

therefore, $$e^b = (p_m/(1-p_m))((1-p_f)/p_f) = OR$$

As the OR approximates to the RR, using the table of actual risks estimated from the model, the estimated relative risks may be calculated.

2.3.5. Anatomical distribution of lesions

Each Case donkey was classified according to the anatomical site of the main sarcoid lesion. The ratios of the distributions of anatomical sites of the lesions on male and female donkeys were examined and tested for statistical significance

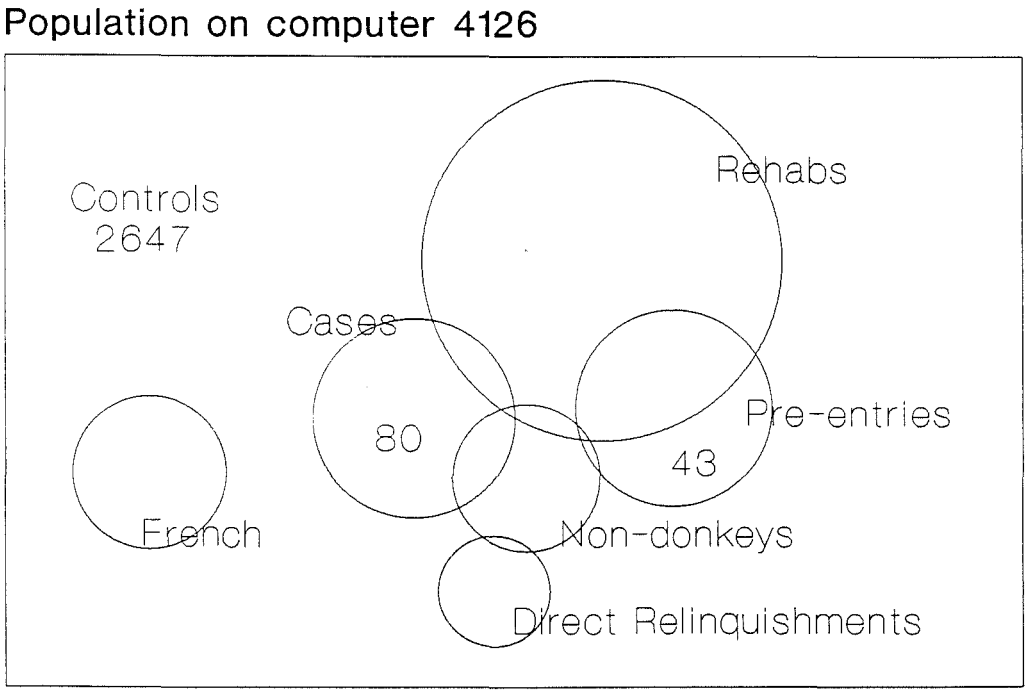
2.3.6. Mortality rates

The dead/live ratios of Case and Control animals were compared and tested for statistically significant difference.

2.4. RESULTS

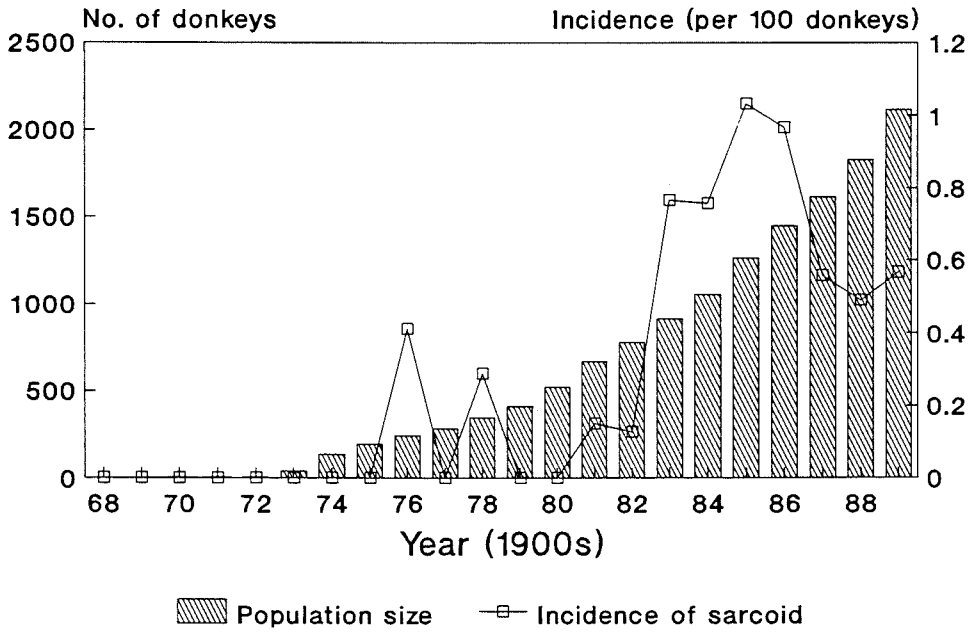
2.4.1. Populations

The records were exported from the Donkey Sanctuary hard disk on 17 July 1990. At this time 4,126 animals were listed on the Sanctuary records. Of these, 2,647 (64.2%) were allocated to the Control group, 80 (1.9%) to the Case group, 43 (1.0%) to the Pre-entry group and 1,356 (32.9%) were excluded from the study. The exclusions included animals other than donkeys and all donkeys that were not currently in, or had ever been temporarily removed from, The Sanctuary. The relationship between the three populations, the exclusions and the total number of animals on record at The Sanctuary is illustrated in Figure 6. The data from the three databases are presented in full in Appendices I, II and III. The ages of dead donkeys are dispalyed in Appendix IV. The prevalence of the sarcoid in all animals at The Sanctuary was 4.4 per 100 animals and 2.9/100 when those presenting with the disease at entry were excluded.



2.4.2. Population growth and incidence of sarcoids

The annual incidence of sarcoids developing at The Donkey Sanctuary is shown in Figure 7. It was not possible to obtain historical data on the number of animals at Rehab or at other locations and complete figures for 1990 were not available. The incidence of sarcoid at The Sanctuary peaked at approximately one case per hundred animals per year in 1985 and 1986. The incidence was a crude estimate due to the fact that the calculation of the animals at risk in any given year was based on the cumulative total of animals entering the Sanctuary minus the cumulative total of dead animals. The population growth is tabulated in Table 2.



2.4.3. Sex

All donkeys in the Control, Case and Pre-entry populations were either female or castrated males. The numbers of animals of each sex in each population are shown in Table 3. The Case population was compared to the Control population and there was a significant difference in the ratio of the sexes ($X^2=6.5$, $df=1$, $p<0.05$), There was no significant difference in the ratio of the sexes between the Case and Pre-entry populations.

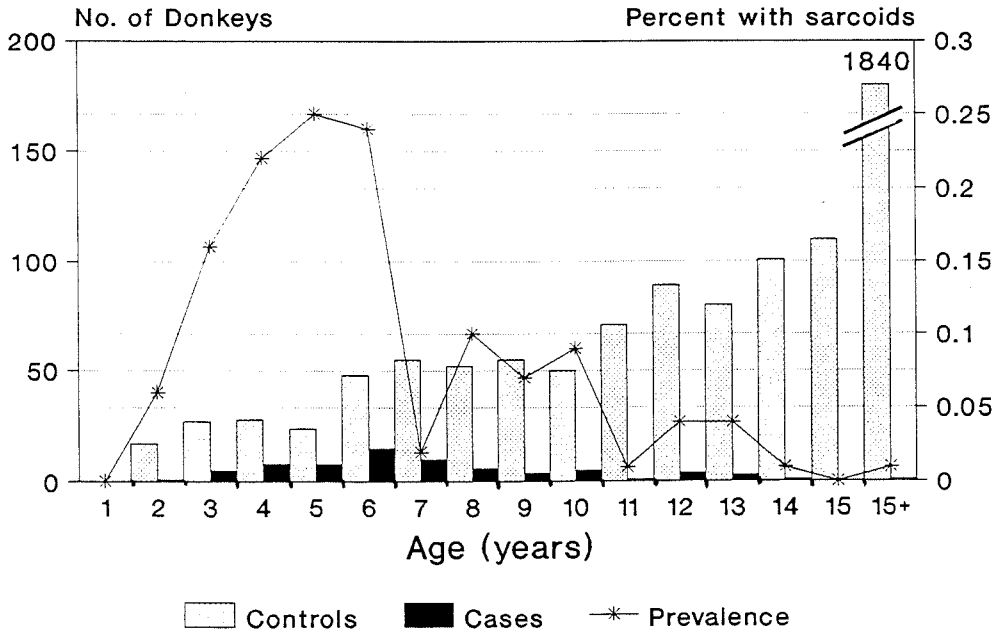
2.4.4. Age at which donkeys entered The Donkey Sanctuary

The summary data and results of the analyses are presented in Tables 4, 5, 6, 7, and 8. It was observed that 8.5% of the 2,647 Controls were aged less than 3 years at entry, 12.8% were aged 3 to 6 years, 11.2% were aged 7 to 9 years, 12.5% 10 to 12 years and 54.9% were aged 12 years or more at entry. This compared with 43.8%, 17.5%, 12.5%, 7.5% and 18.75% in the Case group and 9.3%, 34.9%, 16.3%, 7.0% and 32.6% in the Pre-entry animals. Of all animals less than 3 years of age at entry, 85.2% were Controls, 13.3% were Cases and 1.5% were Pre-entries. In the 3 to 5 year-old group the figures were 92.1%, 3.8% and 4.1% respectively; for the 6 to 8 year-olds, 94.6%, 3.2% and 2.2% respectively; for the 9 to 11 year-olds 97.4%, 1.8% and 0.9% respectively and for the 12 year olds and over, 98.0%, 1.0% and 0.9% respectively. Statistical analysis confirmed that the animals in the Case population were significantly younger at entry than either the Pre-entry ($X^2=16.6$, $df=4$, $p<0.01$) or Control donkeys ($X^2=123.7$, $df=4$, $p<0.001$) and that the Pre-entry donkeys were significantly younger at entry than the Control animals ($X^2=21.5$, $df=4$, $p<0.001$).

2.4.5. Age at which sarcoid tumours were first observed

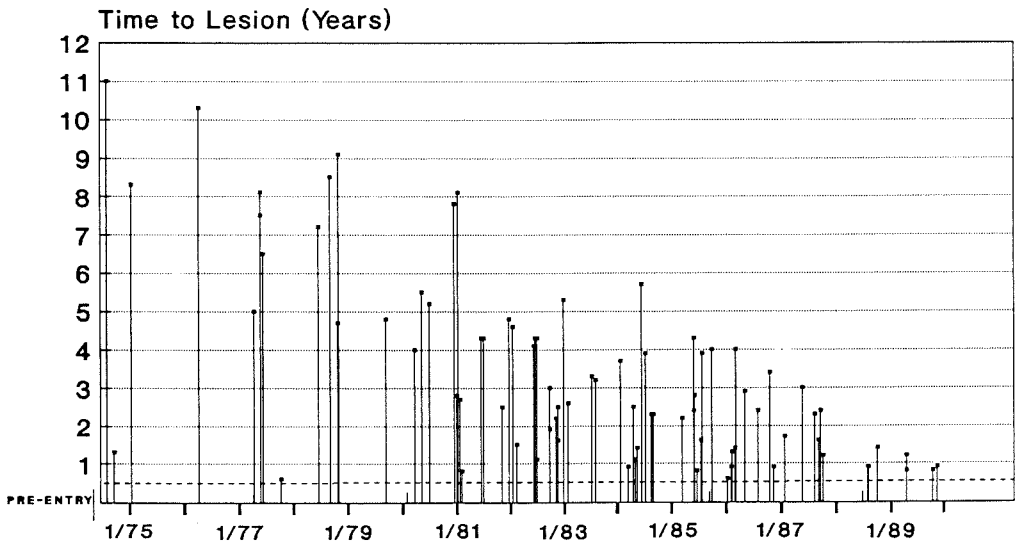
The Pre-entry population was not included in this study as the age at which the disease was first contracted in these animals was not known. The results of the

the highest proportion of clinically affected animals was is in the 2 to 5 year olds, where the disease prevalence was between 15.6 and 25.0 per hundred animals. The prevalence peaked in 5 year-olds and was lowest in the 14 year-olds. The age distributions in the two populations were again highly significantly different ($X^2=249.0$, $df=5$, $p<0.001$) with the Case population being weighted with younger animals.



2.4.6. Time taken from entry to observation of lesion, i.e.,exposure.

The single largest group of animals with sarcoids was that containing those presenting at The Sanctuary with the disease, i.e., the Pre-entry population (exposure = 0 years). These 43 donkeys developed sarcoids before entering The Sanctuary, and so the sarcoids could not possibly be related to risk factors associated with living in The Sanctuary. As a consequence they were excluded from further consideration. With regard to the donkeys in the Case population, animal the length of time between entry and a Case animal being observed with a sarcoid was very variable (Figure 9), ranging from less than a year to in excess of 15 years (Table 11). There was a significant difference ($X^2=52.9$, df=7, $p<0.01$) between the Exposure distributions of the Case and Control populations (Table 12) with the Case population being better represented in the donkeys with a shorter exposure time. The highest disease prevalence (5.9 per hundred) was in the animals that had been at The Sanctuary for between 2 and 3 years (Table 11) Historically, the overall trend was for the Exposure time to become shorter in more recent years (Figure 9). The crude incidence rate for the disease in the population was 0.6 cases per hundred animals per year (619/100,000 animal-years).



2.4.7. Cluster analysis: The Isolation period as a source of infection or dissemination of the disease.

The data and statistical analyses concerning the cluster analysis are presented in Table 13 and depicted graphically in Figure 10. No significant association was demonstrated between the period spent in isolation and the likelihood of developing a sarcoid. One of the 80 Cases and 13 of the 2647 Controls were born at The Sanctuary and never entered the Isolation Unit. Any animal entering the Isolation Unit within three weeks of another may have been allocated to the same Isolation group In the remaining Control population (2634), 977 (37%) donkeys were shown to have been in the Isolation Unit within three weeks of a Pre-entry donkey and 1357 (52%) within three weeks of a donkey that subsequently developed sarcoids (i.e. a Case). In addition, 1654 (63%) of the donkeys were in Isolation within three weeks of either a Pre-entry or a Case. These figures compare with 22 (28%), 43 (54%) and 51 (65%) in the remaining 79 Case donkeys. X^2 analysis of these data sets showed no significant difference between the Case and Control populations. A contiguous association of Cases with Pre-entries, wherein a Case could be linked to a Pre-entry animal by way of another Case donkey, was demonstrable in only 25 (32%) of the 79 Cases.

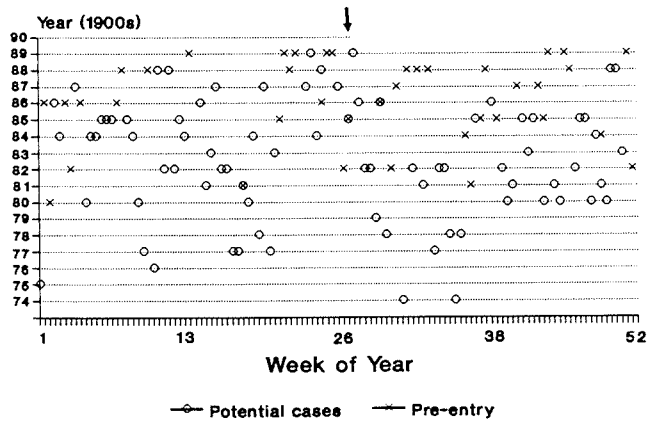
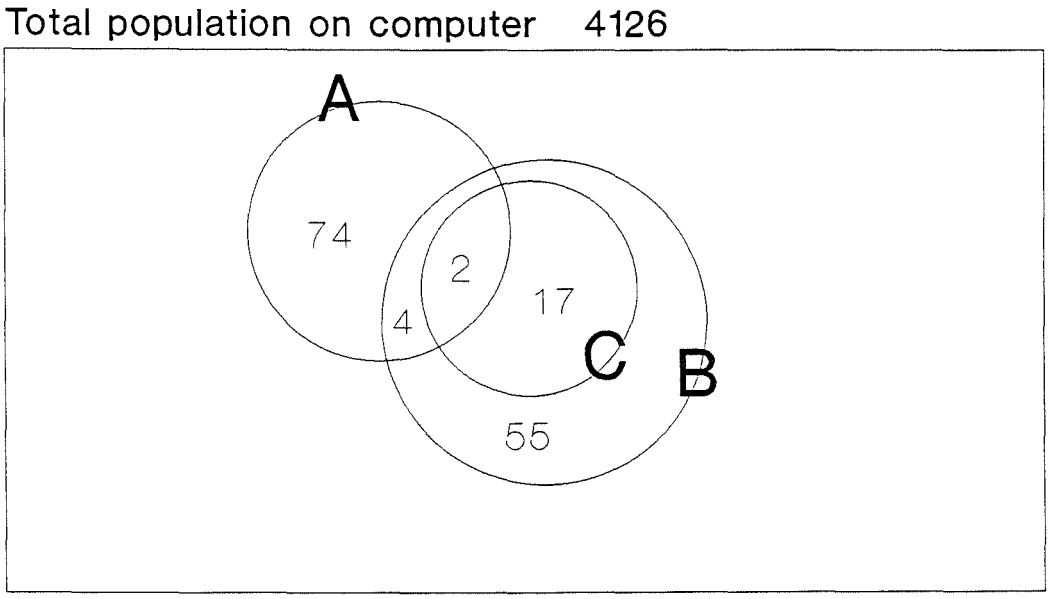


Figure 10. Temporal clustering in the Isolation Unit of Potential Cases (animals that go on to develop sarcoids whilst at The Sanctuary) and Pre-entry animals (those with sarcoids at entry to the

2.4.8. Close in-contacts and relatives

A close in-contact was defined as being a donkey listed in the animal records and having an affinity for a donkey in the Case population. Of the 80 cases, 18 animals were identified as having no close relationship with another animal or animals. The remaining 62 donkeys were listed as having between one and four close relationships and these were defined as either "relation" where there was a known familial relationship, or as "non-relation" otherwise. Forty-two donkeys had one relationship, 11 with relations and 31 with non-relations; 14 animals had two relationships, three with relations and 25 with non-relations; four donkeys had three relationships, three with relations and nine with non-relations; two donkeys had four relationships, two with relations and six with non-relations. In total, there were 78 animals associated closely with sarcoid-affected donkeys, 19 of which were relations and 59 non-relations. In only six of the 78 relationships was the listed animal also sarcoid-affected. Two of the six were relations and four were non-relations of the animals in the Case population. In summary, four of the 59 Case-non-relation relationships were between two Case animals and two of the 19 Case-relation relationships were between two Case animals. This is illustrated in Figure 11.



Unfortunately, it was not feasible to obtain the relationship data for the Control population from the database constucted and so in order to assess the significance of contact with a sarcoid-affected animal, a X^2 analysis was performed, comparing the prevalence of the disease in the in-contact population with the prevalence in the population as a whole, i.e., 6/78 versus 80/2727. The result of this analysis showed that the in-contact animals were significantly more likely to have sarcoids than an animal in the general population ($X^2=5.8$, $df=1$, $p<0.05$).

The comparison of the prevalence of the disease in the relation and non-relation animals, i.e., 2/19 versus 4/59, was performed using Fishers Exact Test because of the small group sizes and it was shown that the probability (P) of the disparity in the disease rates having arisen by chance was $P=0.63$ ("Two-tailed" test). Thus, there was no significant difference between the two very small groups.

2.4.9. The Disease Model

The descriptive statistics described above outlined three selected explanatory variables associated with the likelihood of an animal developing sarcoids. In order to construct a disease model based on the sex, age at entry and the time spent at the Sanctuary before the animals were observed to have a sarcoid (exposure), the data from the Case population were arranged into two tables, one for males, one for females, classifying each animal by age at entry and exposure time (Table 14 and 16). However, due to the sparsity of the data, Tables 14 and 16 were collapsed, condensing the data into Tables 15 and 17. Two disease models were then constructed and tested using the GLIM software, one based upon the numbers of Case animals, the other upon the proportions of affected donkeys in the population.

In the first of these, the distribution was Poisson, due to the sparsity of the data, and the link function log linear. The maximal model, consisting of the main effects and the two-factor interactions, had a scaled deviance of 8.941 with 6

removal of the [sex x age at entry] interaction and the [age at entry x exposure] interaction produced a model with no significant difference to either the maximal model or the full model. The linear predictor of the mimimal model describing the disease at the Sanctuary was selected to be:

1 + sex+ age at entry + exposure + [sex x exposure]

(Scaled deviance = 13.19, df = 15)

The differences between the scaled deviances degrees of freedom of this model and the maximal model were 4.251 and 9, respectively, showing no significant difference from the maximal model or the full model.

When proportions of animals affected in each category were entered, in the creation of a model wherein there was a Binomial distribution and the link function was logit, the full model had a scaled deviance of 8.189 with 6 degrees of freedom. The removal of the [sex x exposure] interaction indicated that, again, this was the most significant two-way interaction and that neither the [sex x entry] nor [entry x exposure] interactions significantly affected the model. The linear predictor for this model was selected to be:

1 + sex+ age at entry + exposure + [sex x exposure]

(Scaled deviance = 16.732, df = 15)

Using the general linear model, the risks were estimated and are displayed in full in Table 18. For example, the ratio of the estimated actual risks of males and females within the the first three years of life at entry and within the first three years of exposure was 0.238/0.121 = 1.967, i.e., being male increased the risk of sarcoids by a factor of approximately 2.0 or being female halved the risk of having sarcoids. In order to put confidence limits on the estimate of the odds ratios, the exponential of the appropriate parameter was taken plus and minus two standard errors (SEs). Thus, for males and females in the present model the estimated difference due to being male, in all groups, was 0.8182. Generating the exponentials plus and minus two SEs produced a lower limit of 0.2204 and an

By taking the reciprocals of these figures, the results were easier to interpret in clinical terms and indicated that being male increases the odds of having sarcoids by a factor of at least 1.132 and possibly by a factor as big as 4.537.

2.4.10. Anatomical distribution of sarcoid tumours

The numbers of male and female Case donkeys and the anatomical site of the primary lesion are summarised in Table 19. Of the 80 Cases, 32.5% had lesions located in the paragenital region, 22.5% had lesion on the head (other than periocular) and 15.0% had periocular lesions. There was a significant difference between numbers of male and female with lesions in the paragenital region ($X^2=9.6$, $df=1$, $p<0.01$) and between male and females with lesions on the ventral abdomen ($X^2=5.8$, $df=1$, $p<0.02$). The single largest group of affected animals was males with paragenital sarcoids (22/80) although when the animals with head and periocular lesions were combined, they accounted for 19 of the 80 animals. Seven of the 80 Cases had lesions at an anatomical site other than the primary site, i.e., had multiple lesions.

2.4.11. Mortality rate

Comparison of the dead/live ratios of the Case and Control populations demonstrated a significant difference ($X^2=4.7$, $df=1$, $p<0.05$) with the Case population having higher ratio (10:70) than the Control population (602:2045). The results are presented in Table 20.

2.5. Discussion

The present study is concerned with animals that develop sarcoids at The Donkey Sanctuary. Eighty such cases were reported, a crude incidence rate of 0.6 cases per hundred animals (619/100,000), a figure significantly higher than the 389/100,000 for *all* benign tumours in a population of army horses reported by the Surgeon

in donkeys kept in the rest of the country but if Dorn and Priesters' figure is an approximate estimate for the general equine population in the United Kingdom, the disease is a cause for concern at The Donkey Sanctuary.

This finding was borne out by the observations of the clinicians at The Sanctuary, who prompted the present study in 1987. Interestingly, the highest incidence of the disease at The Sanctuary was in 1985 and 1986 and it was presumably this increase in case-load, noticed by the clinicians that led to the commission. Although the incidence has decreased since 1986, the economic impact of the disease is considerable, each case requiring perhaps two or three operations to effect a cure which is both expensive and time consuming. However it is the implication to individual animal welfare which justifies the further investigation of the epidemiology of the disease at The Sanctuary.

Despite the fact that there has been no reported sex predisposition, the present study has demonstrated that sarcoids were more likely to affect males than females at The Sanctuary. However, this bias towards males was also noted in those animals presenting with the disease at entry to The Sanctuary and so this may be a feature of donkeys in the population at large in the United Kingdom. Certainly, there is insufficient evidence to suggest that the sex predisposition is a result of management or exposure variables peculiar to The Donkey Sanctuary. More general factors, which may explain the prevalence in male donkeys, will be discussed later in this chapter and in Chapter VIII.

The Control, Case and Pre-entry populations differed significantly in their age at entry. Most animals that developed sarcoids at The Sanctuary entered as young animals; those presenting at The Sanctuary with sarcoids were generally older than Case animals; the majority of Control animals were over 12 years or more. In addition, the age-specific disease prevalence suggests that the sarcoid is a disease of the younger animal and this finding may explain why the donkeys comprising the Pre-entry population were generally older at entry than those that

(Pre-entries), have recovered from the disease or be resistant to the disease (Controls); younger, susceptible animals will develop the disease (Cases).

Given the putative viral aetiology of sarcoids and that an epizootic of sarcoids has been reported (Ragland *et al.* 1966), it might be expected that the animals most closely associated with sarcoid-affected donkeys would be at an increased risk of developing the disease. The possibility that the Isolation Unit may have been a source of infection, or responsible for the spread of the disease, can be refuted on the grounds that the animals that occupied Isolation at the same time as those

i) donkeys with the disease (Pre-entries),

ii) donkeys subsequently developing the disease (Cases),

iii) both (i) and (ii),

were no more likely to develop sarcoids than animals that had not come into contact with these groups. Furthermore, there was a contiguous link between Cases and Pre-entries demonstrable either directly (within 21 days of a Pre-entry), or indirectly (through a Case), in only 32% of cases. It may be concluded that the animals that develop sarcoids at the Sanctuary are unlikely to be acquiring the disease solely due to being associated either with donkeys with sarcoids at entry, or with other animals that develop the disease subsequently, and may therefore have been carriers.

However, other factors encountered at the time of Isolation may play a part in the disease process as evidenced by the significant association between exposure, i.e., time at The Sanctuary, and the development of disease. This may be attributable to some event or environmental factor encountered whilst in Isolation, but although this is a possibility, peak prevalence occurred in animals that had been at The Sanctuary for between 2 and 3 years which is longer than would be expected for the development of most papillomavirus induced tumours (Voss, 1969) Furthermore, there is considerable range of exposure values, with animals

observer related effect and the heavy coat of the donkey and herd management at The Sanctuary may mean that tumours are not being detected until an appreciable size. However, it does seem that the variable time to the development of the disease and the results of the cluster analysis point towards the fact that Isolation is unlikely to be responsible for the propagation of the disease at The Sanctuary. It also calls into question the transmissibilty of the sarcoid in animals kept together in confined accommodation, as suggested by Ragland *et al.* (1966).

Once the animals have left Isolation, the group of donkeys likely to be most at risk, assuming a transmissible agent, are those in contact with affected donkeys on a daily basis. The Sanctuary has identified these so called "donkey-friends", which may be blood relation or non-relation, and the animals are intimately associated together. If the disease is passed from one animal to another, a high prevalence of the disease in the donkey-friends may be expected. Considered as a whole, these close in-contacts were significantly more likely to have sarcoids than animals in the general population and this may indicate that a transmissible agent is involved in the aetiology of the disease, as suggested by Ragland *et al.* (1966). However, the comparison of disease rates in relations and non-relations indicated that the relations were no more likely than the non-relations to have sarcoids and this result is particularly surprising in the light of previous work associating sarcoids with breed and specific leucocyte antigens, i.e., genotype (James, 1968; Lazary *et al.,* 1985; Meredith *et al.,* 1986; Brostrom *et al.,* 1988; Angelos *et al.,* 1988), which would suggest that the relations would show increased susceptibility to the disease when compared to non-relation in-contacts. The analysis is based on a very small data set and although statistical manipulations may be performed to show significance, it would be inappropriate and foolish to attempt to draw any conclusions from these data. In view of the size of the groups, it would be necessary to examine the nature of the familial relationship, e.g., mother-daughter, full-sibling, half-sibling, before commenting further. This is a logical development of

As can be seen, interpretation of the effects of any single variable mentioned above is difficult and it is likely that no single factor will be associated with the disease to the exclusion of all the others. In order to assess the effect of each main variable and the interactions between the variables to the risk of developing sarcoids, a log linear model was employed. The interactions between the variables differ in their significance to the model and the most effective disease model is one in which factors playing no significant role are removed. In the present study, the main variables were judged to be sex, age at entry and exposure and it was these variables that were used in the generalised linear models. The removal of the [sex x entry] and [entry x exposure] interactions did not significantly alter the reliability of the model as indicated by the ratio of the differences of the scaled deviance and degrees of freedom, whilst removal of the [sex x exposure] produced a poorer model. However, although the model constructed from the absolute number of cases was a description of the diseased animals' relationship to each other, it did not take into account the Control population. As it transpired, the model arising from the analysis of the proportions of affected animals compared to the healthy controls was identical to the first model (i.e., only the [sex x exposure] interaction was of significance) and although both models can be used as a means of predicting disease occurrence, the second model is better suited to predicting risks relative to the healthy population, and as such, may be employed as a management tool. This is achieved by the use of the odds ratios and relative risks.

By applying the model, it is evident that males are at best 1.1 times more at risk from sarcoids and at worst 4.5 times more at risk than females. Consultation of the estimated actual risks indicates that in males the disease has the highest rate amongst the 0-3 year olds and followed by the 3-6 year olds in the first three years at The Sanctuary. The disease model used in this manner is directly applicable as a management tool for the clinicians at The Donkey Sanctuary who must identify

each donkey, and judging by the animals which presented for surgical therapy, in many instances the sarcoids are well developed before diagnosis. By consulting the table of risks generated above, the group management of the donkeys can now be adapted to include more regular and more detailed clinical examinations of the animals in the high risk groups, thus increasing the likelihood of earlier diagnosis and treatment. This will have several advantages: Not only will it decrease the scale of trauma of the invasive procedures used to treat sarcoids and thus benefit the donkey, but it will also provide material for investigation from an earlier stage in the disease process.

This chapter has shown that sarcoids at The Donkey Sanctuary are most likely to affect younger, male animals in their first 3 years at The Sanctuary. It has also been shown that, in male animals, the lesions are most frequently observed in the paragenital region. The Isolation Unit does not appear to play a significant role in the spread of the disease but there is an indication that close in-contact animals, by virtue of the fact that they are associated with the Case population are more likely to have sarcoids, than by chance alone. This may indicate that the disease is caused, at least in part by a transmissible agent, or that the animals encounter some event that may pre-dispose them to the disease.

The association of the sarcoid with an area of previous insult to the skin is well documented (Voss, 1969; Ragland *et al.,* 1970a; McMullan, 1982). All of the male animals that enter The Donkey Sanctuary are castrated to prevent them from breeding and to reduce testosterone related behaviour. This is common practice outside The Donkey Sanctuary and the older animals presenting at The Sanctuary are more likely to have been castrated by a previous owner (J.N. Fowler, personal communication). The sarcoids occurring in the paragenital region do not occur exclusively on the site of the scrotal incision and it is unlikely that surgery *per se* is responsible for the introduction of an aetiological agent. However, the trauma of surgical preparation may predipose the site to the post-operative entry of an

reaction (see Chapter VII) demonstrated the presence of papillomaviral DNA on some obstetrical instruments used in the management of patients with human papillomavirus infections, even after the instruments had been sterilised in Savlon or ethanol, thus suggesting that potential pathogens my be transferred from animal to animal at surgery (Ferenczy, Bergeron and Richart, 1989). It is also the case that donkeys and their close friends are castrated at the same time increasing the likelihood of an agent being transferred from a diseased animal to an healthy animal kept in close contact, post-castration.

This theory does not explain why sarcoids affect female donkeys or other parts of the body (perhaps with the exception of the eye which also attracts flies) but it is supported by the age distribution of affected animals as well as the sex, exposure and age at entry variables of the disease model, assuming that the lesions are not noticed until well progressed.

The difference in the mortality rates of Control and Case populations is, perhaps, the most difficult to explain. Animals that have received any type of veterinary attention in the past receive close follow-up inspections by lay and veterinary staff. With specific reference to sarcoids, animals that are diagnosed as having the disease and receive therapy are kept in a group together, allowing close monitoring of tumour recurrence. This group of donkeys is located nearer the centre of The Sanctuary and is much smaller in size than most of the other groups. The change in husbandry may lead to closer attention being paid to the general well-being of these animals, with the result that other life-threatening conditions may be diagnosed and treated earlier than if the donkeys were in a group of 200 animals, thus decreasing mortality rates.

The original reasons for grouping the sarcoid-affected animals together were to facilitate post-operative inspection and follow-up examinations as well as an attempt to limit the spread of the disease, assuming a transmissible agent. However, the herd management system employed by The Sanctuary inevitably

viral, pathogen then the serological status of the animals may provide further epidemiological information with regard to the equine sarcoid and was the basis for the investigations described in the following chapter.

Table 2. Estimated number of donkeys at The Donkey Sanctuary.on 31 December each year, illustrating the rapid growth in the donkey population..

Year	No. of donkeys
1968	1
1969	2
1970	3
1971	3
1972	4
1973	42
1974	138
1975	197
1976	243
1977	283
1978	348
1979	413
1980	523
1981	669
1982	780
1983	915
1984	1056
1985	1260
1986	1449
1987	1615
1988	1832
1989	2116

Table 3. Distribution of the sexes in Control, Case and Pre-entry populations. All male animals were castrated.

	No. of donkeys		
Population	Male	Female	Totals
Controls	1175	1742	2647
Cases	47	33	80
Pre-entries	30	13	43
Totals	1252	1518	2770

Table 4. Summary classification of the Control, Case and Pre-entry populations by age at entry to The Donkey Sanctuary.

Age at Entry (Years)	No.of Donkeys			Totals
	Controls	Cases	Pre-entries	
0.5 to <1	97	8	0	105
1 to <2	69	14	0	83
2 to <3	58	13	4	75
3 to <4	78	5	5	88
4 to <5	129	6	8	143
5 to <6	133	3	2	138
6 to<7	101	5	6	112
7 to <8	99	2	1	102
8 to <9	97	3	0	100
9 to <10	95	3	0	98
10 to <11	158	2	1	161
11 to <12	79	1	2	82
12 to <13	131	2	2	135
13 to <14	76	0	1	77
14 to <15	99	2	0	101
15+	1148	11	11	1170
Total	2647	80	43	2770

Table 5. Summary classification of the Control, Case and Pre-entry populations by age at entry to The Donkey Sanctuary. The age groupings were structured to allow statistical analysis.

Population	Age Group (years)					Total
	0.5 to<3	3 to <6	6 to <9	9 to <12	12 +	
Controls	224	340	297	332	1454	2647
Cases	35	14	10	6	15	80

Table 6. Percentage composition of the Control, Case and Pre-entry populations by age group at entry. Each figure is the percentage contribution of donkeys of a given age at entry to the total number of animals in the specified population.

Population	Age Group (years)				
	0.5 to<3	3 to <6	6 to <9	9 to <12	12 +
Controls	8.5	12.8	11.2	12.5	54.9
Cases	43.8	17.5	12.5	7.5	18.8
Pre-entries	9.3	34.8	16.3	7.0	32.6

Table 7. Percentage composition of age groups at entry by population. Each figure is the percentage contribution of the animals in the specified population to the total number of animals in that age group.

Population	Age Group (years)				
	0.5 to<3	3 to <6	6 to <9	9 to <12	12 +
Controls	85.2	92.1	94.6	97.4	98.0
Cases	13.3	3.8	3.2	1.8	1.0
Pre-entries	1.5	4.1	2.2	0.9	0.9

Table 8. Results of the statistical analyses of the relationships between the age at entry distributions of the Control, Case and Pre-entry populations.

Population	X^2	df	Significance
Case/ Control	123.7	4	$p<0.001$
Pre-entry/Control	21.5	4	$p<0.001$
Case/Pre-entry	16.6	4	$p<0.01$

Table 9. Age-specific disease prevalence of the sarcoid in donkeys at The Donkey Sanctuary. The donkeys in the Case population were classified according to the age at which a sarcoid was first observed. .

Age Group	No.of Donkeys			Prevalence
(Years)	Controls	Cases	Totals	(per 100)
0.5 to <1	0	0	0	-
1 to <2	17	1	18	5.6
2 to <3	27	5	32	15.6
3 to <4	28	8	36	22.2
4 to <5	24	8	32	25.0
5 to <6	48	15	63	23.8
6 to<7	55	1	56	1.8
7 to <8	52	6	58	10.3
8 to <9	55	4	59	6.8
9 to <10	50	5	55	9.1
10 to <11	71	1	72	1.4
11 to <12	89	4	93	4.3
12 to <13	80	3	83	3.6
13 to <14	101	1	102	1.0
14 to <15	110	0	110	0.0
15+	1840	18	1858	1.0
Total	2647	80	2727	

Table 10. Summary classification of the age-specific disease prevalence of the sarcoid in donkeys at The Donkey Sanctuary. The age groupings were structured to allow statistical analysis.

Age Group	0.5 to <3	3 to <6	6 to <9	9 to <12	12 to <15	15+	Totals
Controls	44	100	162	210	291	1840	2647
Cases	6	31	11	10	4	18	80

Table 11. Exposure specific disease prevalence of the sarcoid in the donkeys at The Donkey Sanctuary. The animals were classified according to the number of years spent at The Sanctuary. The Case animals were classified according to the number of years spent at The Sanctuary up until the observation of a sarcoid.

Exposure	No.of Donkeys			Prevalence
(Years)	Controls	Cases	Totals	(per 100)
0.5 to <1	436	12	448	2.7
1 to <2	393	15	408	3.6
2 to <3	272	17	289	5.9
3 to <4	264	9	273	3.3
4 to <5	264	11	275	4.0
5 to <6	174	5	179	2.8
6 to<7	173	1	174	0.6
7 to <8	132	3	135	2.2
8 to <9	139	4	143	2.8
9 to <10	117	1	118	0.8
10 to <11	50	2	52	3.8
11 to <12	56	0	56	0.0
12 to <13	37	0	37	0.0
13 to <14	44	0	44	0.0
14 to <15	38	0	38	0.0
15+	58	0	58	0.0
Total	2647	80	2727	

Table 12. Exposure specific disease prevalence of the sarcoid in the donkeys at The Donkey Sanctuary. The animals were classified according to the number of years spent at The Sanctuary. The Case animals were classified according to the number of years spent at The Sanctuary up until the observation of a sarcoid. The age groupings were structured to allow statistical analysis.

Exposure	0.5 to <1	1 to <2	2 to <3	3 to <4	4 to <5	5 to <10	10 +	Totals
Controls	436	393	272	264	264	735	283	2647
Cases	12	15	17	9	11	14	2	80
Totals	448	408	289	273	275	749	285	2727

$X^2 = 52.9$ ($df = 7$, $p < 0.01$)

Table 13. The numbers and statistical significance of donkeys in the Control and populations housed in the Isolation Unit within three weeks of a Pre-entry donkey, a Case donkey and either a Pre-entry or a Case Donkey.

Population	Link	Controls	Cases	Analysis
With Pre-entries	Yes	977	22	$X^2 = 2.8$
				$df = 1$
	No	1657	57	$p > 0.05$
With Cases	Yes	1357	43	$X^2 = 0.3$
				$df = 1$
	No	1277	36	$p > 0.05$
With Cases or Pre-entries	Yes	1654	51	$X^2 = 0.1$
				$df = 1$
	No	980	28	$p > 0.05$

Table 14. Distribution of male donkeys developing a sarcoid at The Donkey Sanctuary (i.e., male Cases). The animals are classified by the year of life during which they entered The Sanctuary and the year after entry during which the lesion was first observed.

Age at Entry	Exposure (Years)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15+
1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
2	2	4	2	1	4	1	0	0	0	0	0	0	0	0	0	0
3	2	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0
5	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
7	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
8	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
15+	2	1	1	0	1	1	0	0	0	0	1	0	0	0	0	0

Table 15. Distribution of male donkeys developing a sarcoid at The Donkey Sanctuary (i.e., male Cases). The animals are classified according to the year of life during which they entered The Sanctuary and the year after entry during which the lesion was first ob served. The figures in brackets are the numbers of Control male donkeys in the same category.

Age at Entry (Years)	Exposure (Years)		
	0.5 to <3	3 to <6	6 and over
0.5 to <3	16 (44)	7 (25)	0 (40)
3 to <6	6 (57)	1 (32)	1 (60)

Table 16. Distribution of female donkeys developing a sarcoid at The Donkey Sanctuary (i.e., female Cases). The animals are classified by the year of life during which they entered The Sanctuary and the year after entry during which the lesion was first observed.

Age at Entry	Exposure (Years)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15+
1	0	0	0	1	0	1	0	1	2	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	2	2	1	0	1	1	0	0	0	0	0	0	0	0	0
4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	2	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
6	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
10	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
11	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
15+	0	1	0	0	2	0	0	1	0	0	0	0	0	0	0	0

Table 17. Distribution of female donkeys developing a sarcoid at The Donkey Sanctuary (i.e., female Cases). The animals are classified according to the year of life during which they entered The Sanctuary and the year after entry during which the lesion was first observed. The figures in brackets are the numbers of Control female donkeys in the same category.

Age at Entry (Years)	Exposure (Years)		
	0.5 to <3	3 to <6	6 and over
0.5 to <3	4 (39)	4 (22)	4 (54)
3 to <6	3 (57)	3 (34)	0 (100)
6 to <9	2 (39)	1 (41)	1 (85)

Table 18. Estimated actual risks, generated by GLIM, for male and female donkeys at a given age of entry and length of exposure. The estimated actual risks for female donkeys are given in brackets.

Age at Entry (Years)	Exposure (Years)		
	0.5 to <3	3 to <6	6 and over
0.5 to <3	0.238 (0.121)	0.201 (0.157)	0.024 (0.069)
3 to <6	0.082 (0.038)	0.067 (0.050)	0.007 (0.021)
6 to <9	0.070 (0.032)	0.057 (0.043)	0.006 (0.018)
9 and over	0.020 (0.009)	0.016 (0.012)	0.002 (0.005)

Table 19. Numbers of male and female donkeys, of the Case population, categorised by the anatomical site of the primary sarcoid tumour.

Sex	Anatomical site						Totals
	Peri-ocular	Other head	Ventral abdomen	Para-genital	Limb	Other	
Male	7	12	2	22	4	0	47
Female	5	6	9	6	4	3	33
Totals	12	18	11	28	8	3	80

Table 20. Numbers of donkeys alive and dead in the Control and Case populations.

Population	Dead	Live	Totals
Controls	602	2045	2647
Cases	10	70	80
Totals	612	2115	2727

CHAPTER III

SEROLOGICAL STUDIES: DETECTION OF ANTIBODIES TO PAPILLOMAVIRAL ANTIGENS IN THE SERA OF DONKEYS WITH AND WITHOUT SARCOIDS.

CHAPTER III

SEROLOGICAL STUDIES: DETECTION OF ANTIBODIES TO PAPILLOMAVIRAL ANTIGENS IN THE SERA OF DONKEYS WITH AND WITHOUT SARCOIDS.

3.1. INTRODUCTION

Investigation into the humoral response of animals to foreign antigen can provide information regarding exposure to the antigen, the prevalence of infection, but not necessarily clinical disease, in a population. Serological assays to measure the prevalence of HPV infection have been hindered by the lack of a adequate supply of viral antigen (Galloway, 1990). There are three reasons for this shortage of antigen: First, genital HPV lesions produce few virus particles (Pfister, 1984); secondly, non-genital lesions, such as plantar warts, which produce large amounts of virus, are seldom removed surgically; thirdly, there is currently no *in vitro* method of propagating large quantities of papillomavirus. These difficulties have recently been overcome, to an extent, by the expression of HPV ORFs in bacteria, and the fusion proteins produced in this manner have been used as target antigens in serological assays. This technique is being used to conduct seroepidemiological investigations into the prevalence of HPV infection (Galloway and Jenison, 1990; Jenison, Yu, Valentine, Koutsky, Christiansen, Beckmann and Galloway, 1990; Steger, Olszewsky, Stockfleth and Pfister, 1990).

Unlike HPV infections, BPV-1 and 2 lesions produce large amounts of virus and the tumours are readily available from abbatoirs. It has been shown that polyclonal antibodies raised against intact PV virions cross-react with closely

Olson, Pass, Lancaster and Shah, 1980). Genus-specific, cross-reactive and type-specific epitopes have been identified by the use of monoclonal antibodies raised to disrupted BPV-1 (Gorra, Lancaster, Kurman and Jenson, 1985; Nakai, Lancaster, Lim and Jenson, 1986 Cowsert, Lake and Jenson, 1987). Gorra *et al.*, (1985) and Nakai *et al.*, (1986) demonstrated a BPV-1 specificity in some monoclonal antibodies raised to disrupted BPV-1. Cowsert *et al.* (1987) defined the BPV-1 epitopes as external conformational, external linear and internal linear depending upon reactivity with intact virions alone, intact and disrupted virion or disrupted virion alone. In addition, as in HPV research, the cloning and expression of BPV-1 (Pilacinski, Glassman, Krzyzek, Sadowski and Robbins, 1984) and BPV-2 (Jarrett *et al.*, 1991) ORFs have allowed the production of large amounts of viral protein. This has provided an additional tool for the detection of antibodies to BPV infection as well as providing viral protein in sufficient quantities for prophylactic and therapeutic vaccination studies (Pilacinski *et al.* 1986, Jarrett *et al.* 1991).

The experiments in this chapter investigated whether or not donkeys, with or without sarcoids, at The Donkey Sanctuary had circulating antibody directed against papillomaviral antigens and, secondly, if the antibody detected was capable of neutralising the transforming activity of BPV-2 *in vitro.*

Polyacrylamide gel electrophoresis (PAGE) formed the basis of the first part of the serological investigation. The first experiments assayed donkey sera from animals at The Donkey Sanctuary against the antigens present in the disrupted BPV-2 virion. The virus used as the target antigen was extracted from BPV-2 lesions by the rate zonal method described in Chapter IV may have contained small amounts of contaminating bovine cellular material. It is also known that the molecular weights of cytokeratins in mammalian skin are between 40 to 70kDa (Franke, Schmid, Osborn and Weber, 1978; Franke, Appelhans, Schmid, Freudenstein, Osborn and Weber, 1979; Hoshimoto, Eto, Matsumoto and

1984). Thus, in order to rule out the possibility that the antibodies detected by immunoblot were directed against a bovine cytokeratin, the donkey sera were also assayed against the L1 fusion protein of BPV-2. The immunoblot assays (Jarrett *et al.,* 1990b) used in the two experiments were similar. A brief description of the standard gel casting technique, modified from Laemmeli (1970), is given and the formulations listed.

The latter part of this chapter describes the assay of the donkey sera for the presence of neutralising antibody against BPV-2. The presence of neutralising antibodies in serum samples was measured in a quantal assay by the ability of the sera to inhibit the transformation of bovine fibroblasts by BPV-2 when the serum was mixed with a known amount of virus, before the addition of the virus to the indicator cells. The methodology (Jarrett *at al.,* 1990a) is described below.

3.2. MATERIALS AND METHODS

3.2.1. Source of donkey sera

The donkeys at The Donkey Sanctuary are routinely bled in order to monitor haematological and plasma biochemical parameters. All samples of serum used in this study were obtained at the same time as the routine samples for The Sanctuary's health monitoring programme. Ten ml of blood were collected and allowed to clot. Serum was removed from the sample and centrifuged briefly in a bench-top centrifuge to pellet any remaining erythrocytes. The serum was stored in 1ml aliquots at -20^{o}C, until required.

3.2.2. Detection of antibody against papillomaviral antigens by immunoassay.

3.2.2.1. Preparation of antigen

Two different sources of antigenic material were prepared for polyacrylamide gel electrophoresis.

Disrupted virion: Three hundred ul of BPV-2 virions, purified by the rate zonal

(Laemmeli 1970) heated to 95^{o}C and cooled on ice immediately prior to electrophoresis.

L1 fusion protein: The BPV-2 L1 ORF, molecularly cloned in two fragments into pUR vector series (Jarrett *et al.* 1991), was used to produce two polypeptides: a truncated N-terminal half and a C-terminal half of the L1 protein. The following is an account of the method used to produce sufficient quantities of the protein for use in the immunoblot assays. Two hundred ul of competent *E. coli* JM109 cells were inoculated into 1ml L broth and incubated overnight at 37^{o}C with shaking at 225*g*. Approximately 100ul of this overnight culture was seeded into 100ml L broth and the incubation continued for a further 2 hours. The bacteria were then pelleted by centrifugation in a Beckman J2-21 centrifuge at 2000*g* at 4^{o}C. The pellet was resuspended in one fifth volume of 50mM calcium chloride and allowed to stand on ice for 20 minutes. The centrifugation was repeated and the pellet resuspended in one twentieth volume 50mM calcium chloride solution. The competent cells were then aliquoted into 100ul volumes and stored for no longer than 48 hours at 4^{o}C.

For each plasmid containing a fragment of the L1 ORF of the BPV-2 genome, the plasmid DNA was diluted to a concentration of 1ng/ul in TE buffer. Ten ul of DNA was added to 100ul of competent cells and allowed to stand on ice for 20 minutes. The cells were then heat shocked for 90 seconds at 42^{o}C with shaking followed by the addition of 400ul L broth and the suspension incubated at 37^{o}C for 60 minutes. Fifty to 100ul were then plated out on to agar plates containing ampicillin at a concentration of 100ug/ml. The plate was incubated at 37^{o}C overnight in an inverted position.

A single bacterial colony from the overnight culture was picked up with a platinum loop and inoculated into 5ml L broth containing ampicillin at a concentration of 100ug/ml. This was incubated at 37^{o}C overnight as before. One hundredth of this culture was seeded into 400ml L broth with ampicillin and the

continued for 30 minutes. The bacterial pellet was recovered following centrifugation at 5000*g* for 10 minutes at 4°C and resuspended in 14.4ml lysozyme buffer (25 per cent sucrose, 50 mM Tris-HCl, pH 8.0). To this suspension, 1.3ml of lysozyme solution (10mg/ml in lysozyme buffer) was added and the mixture stored on ice for 15 minutes. The bacteria were then lysed by the addition of 6ml of lysis buffer (1 per cent Nonidet P40, 0.1 per cent sodium deoxycholate, 0.1M sodium chloride, 10mM Tris-HCl (pH 8.0) into 30ml corex tubes. The bacterial debris was pelleted by centrifugation at 10000*g* for 10 minutes and the supernatant retained. The pellet was washed with guanidinium hydrochloride in 1M sodium chloride and 1 per cent Triton X-100. Centrifugation was repeated and the pellet washed in 10mM Tris-HCl (pH 8.0) a further two times. The protein pellet was resuspended in 15ml of sample buffer and heated to 98°C for 15 minutes. The two portions of the L1 encoded protein were combined in equal quantities and were referred to as the L1 fusion protein.

3.2.2.2. SDS-Polyacrylamide Gel Electrophoresis

Acrylamide/Bis : 37.5g acrylamide and 1.0g BIS dissolved in 125g distilled water.

	Running Gel (10 per cent)	*Stacking Gel (5 per cent)*
Acrylamide/Bis	12g	2g
Buffer	9ml	3ml
Distilled water	18ml	7ml
TEMED	40ul	30ul
Ammonium persulphate(10%)	300ul	60ul

	Running gel buffer	*Stacking gel buffer*
Tris base	18.2g	5.9g
SDS	0.4g	0.4g

Electrophoresis buffer (10x): 63.2g Tris base, 10.0g SDS, 40.0g glycine dissolved in 800ml distilled water. Volume made up to 1l.

Blotting buffer: 9.09g Tris base, 43.2 g glycine dissolved in 2800ml distilled water. Volume made up to 3 litres. To use, 1600ml buffer was diluted with 400ml methanol.

Sample buffer: 1.5g Tris base, 2.1g SDS, 10ml gycerol, 5ml mercaptoethanol, 0.1ml bromophenol blue dissolved in 80ml distilled water. Volume was adjusted to 100ml.

The Protean II Gel Electrophoresis System (Biorad) was used according to the manufacturer's instructions for all SDS-PAGE procedures. Two 16cm x 20cm and 18.2cm x 20cm glass plates were washed with alcohol. The plates were sandwiched together, so that the lower edges were flush, and screw clamps applied. The unit was fitted into the casting slot over a rubber strip to form a seal which was tested by adding water. A 10 per cent polyacrylamide slab gel was poured and left to polymerise for a minimum period of 30 minutes. Ammonium persulphate (APS) initiated the polymerisation whilst N,N,N',N'-tetramethyl ethylene diamine (TEMED) catalysed the reaction. A small amount of butanol was added to remove the bubbles at the air/gel interface and to exclude air, allowing polymerisation. Once set, the butanol was washed off with distilled water and the water drawn off with a syringe and needle. A 5 per cent stacking gel was poured and a preparative well comb with individual marker well was inserted. The different pH and gel pore size caused the proteins to stack producing a sharp band at the start of the running gel at which point resolution of the proteins started. The gel was allowed to polymerise for a minimum period of 30 minutes after which time the comb was removed and any unpolymerised gel mixture removed by washing with distilled

the upper chamber. The whole assembly was placed in the buffer tank and electrophoresis buffer added to the upper and lower chambers, ensuring the gel plates were covered to a depth of approximately 2cm. Air bubbles along the bottom of the plate were removed by a syringe. Sample buffer was added to all samples and standard molecular weight markers were run alongside the samples. A linear plot of the distance migrated (electrophoretic mobility) against known molecular weight was plotted on semi-logarithmic graph paper, enabling the weights of sample proteins, run simultaneously to the standards, to be estimated by interpolation. Electrophoresis was carried out at a constant 30mA for 3 to 6 hours or until the dye front was 0.5cm from the bottom of the gel. Following electrophoresis, the gel was allowed to equilibrate in blotting buffer for 30 minutes. A vertical strip of the gel was removed and stained for one hour in Coomassie blue (0.2 per cent Coomassie Blue, 50 per cent methanol, 12 per cent acetic acid) on a rocking platform. This was followed by several washes in destain solution (7 per cent acetic acid) to reveal the banding pattern of the proteins. The gel strip was then dried under vacuum on a Biorad Model 483 gel dryer at 80^{O}C for 2 hours.

In order to allow the detection of specific proteins by immunochemical techniques, it is convenient to transfer the protein profile to a solid support. Applying an electric current forcibly elutes the proteins from the gel and binds them to a membranous support such as nitrocellulose acetate (Towbin, Staehelin and Gordon, 1979). The Trans-Blot Cell (Biorad) was used, transferring the proteins from cathode to anode.

Assembly of the gel holder was carried out in a tray filled with blotting buffer ensuring the pads, paper and nitrocellulose acetate membrane were all saturated with the buffer. The gel holder was assembled from cathode to anode comprising, in order, `Scotchbritepad' (3M), filter paper (3MM chromatography Whatman), gel with orientation marker, nitrocellulose acetate (Hybond-C,

hands only. The holder was then inserted into the blotting tank and approximately 3l of blotting buffer added. Transfer was performed at 4^{O}C overnight at a constant 135 mA with mixing. Non-specific adsorption sites were blocked by immersing the membrane in Blotto (5 per cent (w/v) milk powder in PBS (pH 7.4)) for 3 hours at room temperature with agitation. After blocking, the membrane was dried at 80^{O}C for 2 hours, cut into numbered strips and stored at -20^{O}C between two sheets of filter paper and encased in Parafilm.

3.2.2.3. Immunoblot assay

The strips of nitrocellulose were placed in individual 15ml Falcon tubes and incubated at 4^{O}C overnight with donkey sera diluted 1:50. in Blotto. Rabbit antiserum (conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP)) to BPV-2 was used (1:1000) as a positive control. The strips were then washed for three 10 minute periods in PBS (pH 7.4)/Tween 20 (200:1) and then incubated for 90 minutes at room temperature with goat anti-donkey IgG (1:500). The washing was repeated and the strips incubated with a horseradish peroxidase HRP or AP conjugated rabbit anti-goat IgG (1:1000) in Blotto for 90 minutes at room temperature. The strips were then washed again and those which had been incubated with a secondary antibody conjugated to HRP were incubated in a solution containing 0.05 per cent (w/v) 3,3' diaminobenzidine (DAB) and 1 per cent (v/v) hydrogen peroxide in Tris buffer. The development of a signal was monitored and the reaction stopped by the addition of tap water. When a secondary antibody conjugated to AP was used, the strips were equilibrated in AP buffer containing 100mM sodium chloride, 5mM magnesium chloride and 100mM diethanolamine (pH 9.5). Two stock solutions containing 0.5g nitroblue tetrazolium (NBT) in 10ml of 70 per cent dimethylformamide and 0.5g bromochloroindolyl phosphate (BCIP) in 10ml of 100 per cent dimethylformamide were prepared. To 10ml of AP buffer was added 66ul of NBT stock and 33ul of

to 30 minutes and was stopped by the addition of PBS (pH 7.4) containing 20mM EDTA. Selected serum samples were pre-incubated with BPV-2 virion, L1 fusion protein, uninduced *E. coli* proteins or beta-galactosidase at 4^{o}C overnight and then assayed against the appropriate target antigens as described above in order to demonstrate that any antibody detected could be adsorbed by pre-incubation with specific antigen resulting in a reduced signal intensity.

3.2.3. Detection of neutralising antibodies.

In the transformation and neutralisation assays described below (Jarrett *et al.*, 1990a), the normal additives of sodium bicarbonate (3.7g/l) and L-glutamine (0.396g/l) were added to the Dulbecco's modification of Eagle's medium used in the assays.

3.2.3.1. Transformation assay

Skin fibroblasts were obtained from bovine fetuses and were tested for their ability to exhibit a transformed phenotype when infected with BPV-2. Suitable primary cell cultures were amplified and stored. A large stock virus preparation from bovine skin lesions (Section 4.2.) was prepared and tested on the bovine fetus cells to determine the 50 per cent tissue culture transforming dose ($TCTD_{50}$). The cells were plated out into a 24-well culture plate at a density of $3x10^4$ cells per well in 1ml of DMEM containing 10 per cent fetal calf serum (FCS). The medium was removed 24 hours later and replaced by DMEM containing 10 per cent FCS and doubling dilutions of the viral stock preparation down to a concentration of 1:1280. Each dilution was prepared in sextuplet. The virus/medium mixture was incubated with the cells for overnight at 37^{o}C and in 5 per cent carbon dioxide. The medium was then removed completely and replaced with DMEM containing 10 per cent FCS and gentamycin (50ug/ml). The medium was changed every 48 hours until transformation was observed.

3.2.3.2. BPV-2 neutralisation assay.

The donkey sera were filter sterilised through a 0.2um filter and complement inactivated by incubation at 55°C for 30 minutes. Doubling dilutions of each serum sample were prepared in DMEM (without FCS) down to a dilution of 1:320. BPV-2 at a concentration twice that of the $TCTD_{50}$ was added to the serum dilutions and, after mixing, the mixtures incubated overnight at 4°C. The virus/serum mixtures were then added to the indicator cells which had previously been inoculated into a 24-well culture plate as described for the transformation assay (section 3.2.3.1.). One ml of the virus/serum mixture was added to each well and allowed to remain in contact with the cells overnight at 37°C in an atmosphere containing 5 per cent carbon dioxide. The virus/serum mixture was removed and replaced with DMEM containing 10 per cent FCS and gentamycin (50ug/ml). This was replaced every 48 hours for 14 days or until the positive control cells exhibited transformation. The medium was then decanted from the wells and the cells washed gently in phosphate buffer (pH 6.8). The cells were fixed in methanol for a minimum period of 15 minutes and then stained with prefiltered Giemsa stain for 30 minutes. The wells were then washed gently with water and air dried. Assays were read using an inverting microscope. The controls used in the neutralisation assays were: 1:10 dilution donkey serum with DMEM (negative); DMEM alone (negative): transforming titre of BPV-2 virus in DMEM (positive). One ml of each of these controls was used in an identical manner to the virus/serum mixtures.

3.3. RESULTS

3.3.1. Immunoblot assay

The results of the immunoblot assays are summarised in Table 21 and presented in full in Table 22 and the appearance of the nitrocellulose strips after the assays is displayed in Figure 12. In total, 45 serum samples from 39 donkeys were assayed for the presence of antibody. When animals were sampled on more than one

Of the 33 sera from 27 donkeys assayed against disrupted virion, 32 samples from 26 donkeys contained antibodies against antigens 55 and 65kDa in size (Figure 12). Fourteen of these donkeys had no evidence or previous history of sarcoids. All samples from sarcoid-affected animals were positive for antibody to the two antigenic bands. One of the fourteen healthy donkeys was a pre-colostrum neonate foal and was negative for antibody reactive with either antigen band. A demonstrable reduction in signal intensity was obtained by pre-incubating positive samples with whole BPV-2 virion. Some of the serum samples contained antibody to a 76kDa fragment, but this was an inconsistent finding and was dependent upon the blot that was used for the assay, rather than the serum sample. The control sample (antisera to BPV-2 raised in a rabbit) only reacted with a broad antigenic band approximately 55kDa in size.

Of the 45 samples, 43 sera from 37 donkeys were assayed against the molecularly cloned L1 fusion protein. Thirty-eight of the samples from 32 donkeys contained antibody reactive with antigenic bands which corresponded approximately with the N-terminus (144kDa) and C-terminus (139kDa) fragments of the L1 protein. All samples, bar one, from sarcoid-affected donkeys were positive. Eighteen of twenty-two healthy donkeys were also positive for antibody. Four donkeys, all healthy animals, including a serum sample from a pre-colostrum neonate foal, were negative for antibody against both fragments (Figure 12). All samples assayed were either positive or negative for both fragments. Samples incubated with the L1 fusion protein prior to assay demonstrated a reduced intensity of signal but it was not possible to remove the signal absolutely. Pre-incubation of the sera with uninduced *E. coli* or beta-galactosidase did not reduce the signal intensity in the region of the fusion proteins.

Thirty-two of the samples were assayed against both fusion protein and disrupted virion and results were consistent between the two assays, i.e., samples positive for antibody against the L1 fusion protein were also positive for antibody

BCIP/NBT/AP detection systems was noted in 19 samples assayed using both systems (Figure 12).

Table 21. Summary of immunoblot assays for the presence of antibodies, reactive with one or more papillomaviral antigens, in serum samples from donkeys at The Donkey Sanctuary.

	L1 fusion protein		Disrupted virion	
	Sera	Donkeys	Sera	Donkeys
No. Assayed	43	37	33	27
No. Positive	38	32	32	26
% Positive	88	86	97	96

Table 22. Immunoblot assays for donkey serum antibody, in donkeys, reactive with the two fragments of the molecularly cloned L1 fusion protein and against disrupted virion (continued).

Animal	Antibody vs. L1 fusion protein		Antibody vs. disrupted virion		Disease
No.	N-terminus	C-terminus	55kDa	65kDa	Status
2087i	+	+	+	+	Sarcoid
2087ii	+	+	+	+	Sarcoid
3590i	+	+	+	+	Sarcoid
3590ii	+	+	+	+	Sarcoid
2074	+	+	+	+	Healthy
2077i	+	+	+	+	Sarcoid
2077ii	+	+	+	+	Sarcoid
2077iii	+	+	+	+	Sarcoid
513	+	+	N/A	N/A	Healthy
514i	+	+	+	+	Sarcoid
514ii	+	+	+	+	Sarcoid
2530	-	-	N/A	N/A	Healthy
3075	+	+	+	+	Sarcoid
3472i	+	+	+	+	Healthy
3472ii	+	+	+	+	Healthy
3459	+	+	+	+	Sarcoid
3675	+	+	+	+	Healthy
2073	+	+	+	+	Healthy
863	-	-	N/A	N/A	Sarcoid
864	+	+	+	+	Healthy
865	-	-	N/A	N/A	Healthy
3529	+	+	+	+	Sarcoid
1708	+	+	+	+	Sarcoid
1709	+	+	N/A	N/A	Healthy

N/A: not assayed.

Table 22. (continued) Immunoblot assays for donkey serum antibody, in donkeys, reactive with the two fragments of the molecularly cloned L1 fusion protein and against disrupted virion.

Animal	Antibody vs. L1 fusion protein		Antibody vs. disrupted virion		Disease
No.	N-terminus	C-terminus	55kDa	65kDa	Status
3037	+	+	+	+	Sarcoid
2428	+	+	+	+	Sarcoid
3485	+	+	+	+	Healthy
3674	+	+	+	+	Healthy
2529	+	+	+	+	Sarcoid
2085	+	+	+	+	Healthy
3676	+	+	+	+	Healthy
3470	+	+	+	+	Sarcoid
876	+	+	+	+	Sarcoid
877	+	+	N/A	N/A	Healthy
878	+	+	+	+	Healthy
3672	+	+	N/A	N/A	Healthy
1131	-	-	N/A	N/A	Healthy
3486	+	+	+	+	Healthy
2075	+	+	N/A	N/A	Healthy
3776	N/A	N/A	+	+	Healthy
3579	N/A	N/A	+	+	Healthy
2515	+	+	N/A	N/A	Sarcoid
4107	-	-	-	-	Pre-colostral
513	+	+	N/A	N/A	Healthy
4044	+	+	N/A	N/A	Healthy foal

N/A: not assayed.

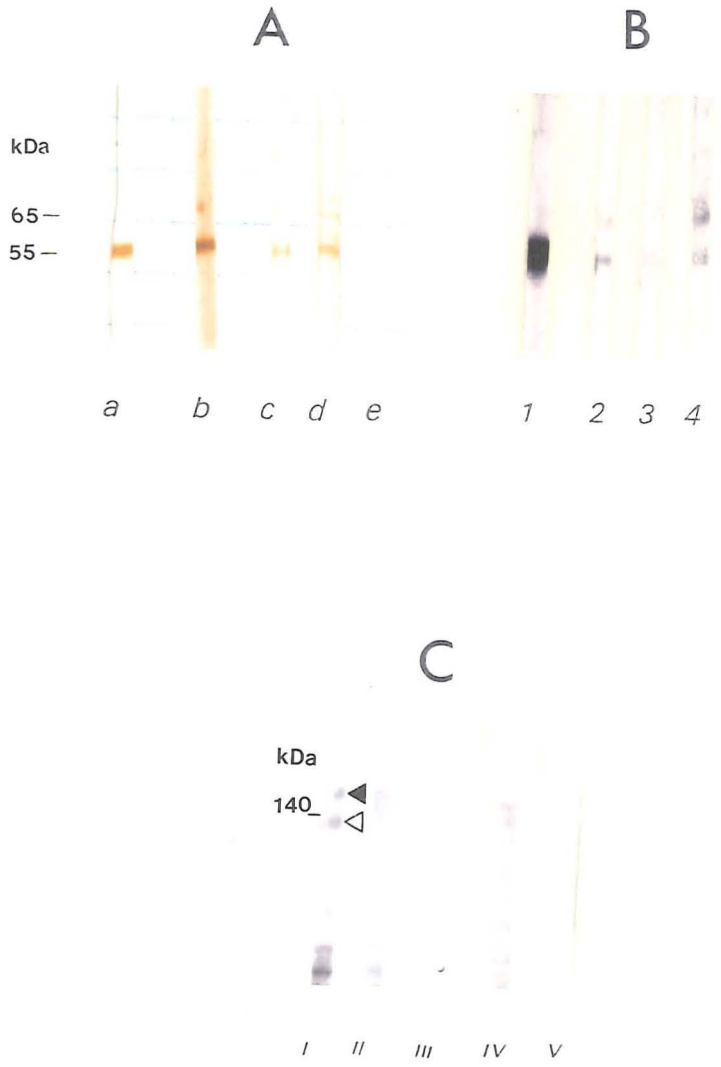


Figure 12. Nitrocellulose strips after immunoblot assay using HRP/DAB (A) and AP/BCIP/NBT (B and C) detection systems. The antigenic targets used were disrupted BPV-2 virion (A and B) and the combined N- terminus (◀) and C-terminus (◁) fusion proteins encoded by L1 open reading frame of BPV-2 (C). The test sera were:(a and 1) antisera to BPV-2 raised in a rabbit; (*i*) antisera to L1 fusion proteins raised in a rabbit; (b, 2 and *ii*) sarcoid-affected donkey serum;(d, 4 and *iv*)healthy donkey serum; (e and v) neonate (pre-colostrum) donkey serum; (c) sample from b preadsorbed with BPV-2 virion; (*iii*)sample from *ii* preadsorbed with L1 fusion protein.

3.3.2. Transformation assay

The $TCTD_{50}$ was found to be 1:320 and in all subsequent neutralisation assays the virus stock was diluted 1:160.

3.3.3. Neutralisation Assay

The sera assayed for the presence of neutralising antibody to BPV-2 were the same as those assayed in the immunoblot experiments. A total number of 45 sera from 39 donkeys were screened and all were found to be negative for neutralising antibody to BPV-2. Seven assays were performed using four different indicator primary cell cultures of bovine fibroblasts, viz., F6 CL3 (twice), F29 P6 (twice), F18 P9 (three assays). The assays lasted between 14 (F18 P9) and 21 days (F6 CL3). All cultures were at passage 12 or less. Assays one to three produced equivocal results as there was little discernible difference between negative and positive controls. Assay four was abandoned after 16 days due to fungal contamination of the cells in the control wells. In assays five, six, and seven (cell line F18 P9) transformation of the cells occurred in all wells which had contained the donkey sera and virus mixture (Figure 13). Transformation did not occur in cells that had been incubated with medium alone or donkey serum alone.

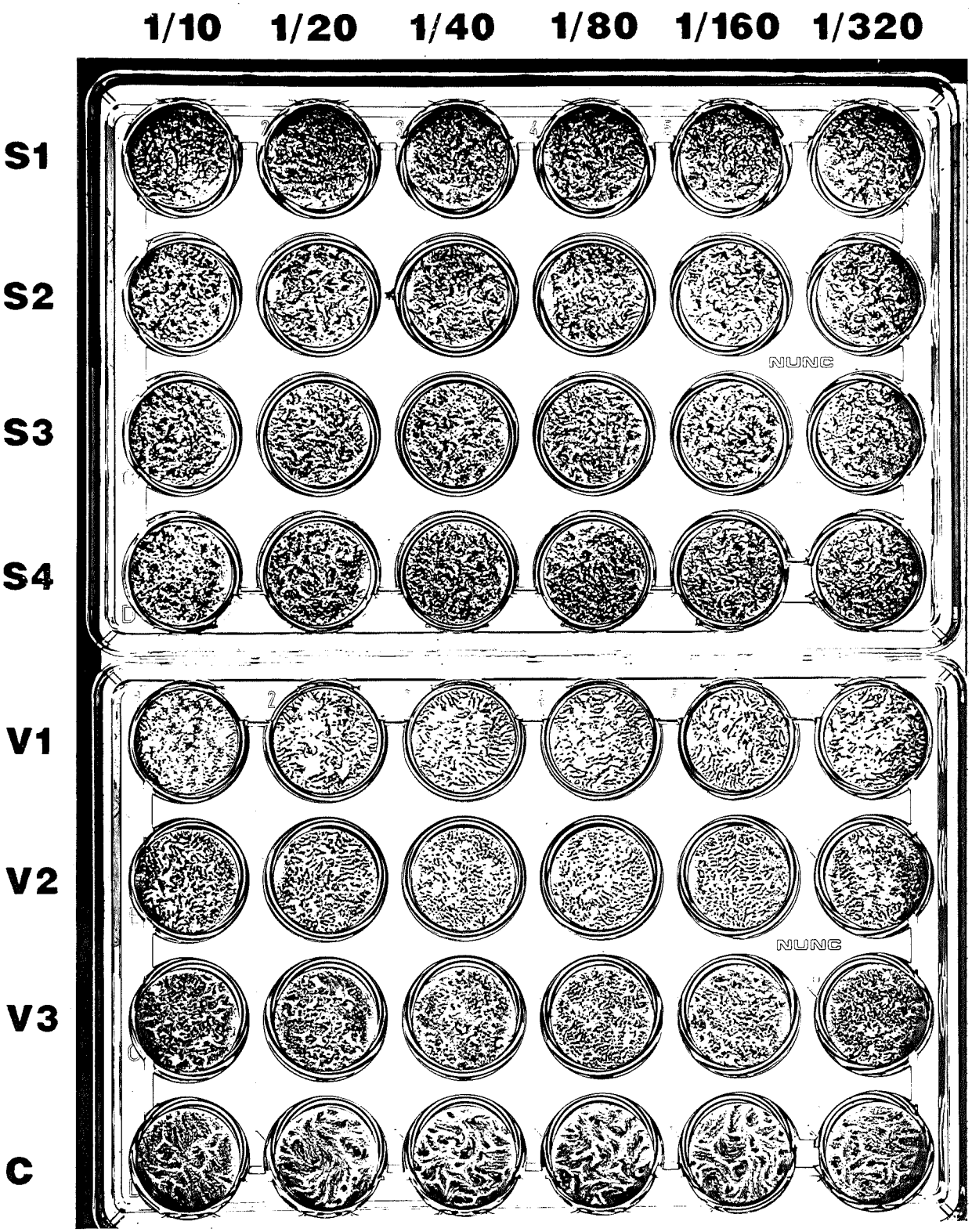


Figure 13. Two 24-well plates after the neutralisation assay. Incubation was at 4°C over night with the following mixtures: S1, wells containing bovine fetal fibroblasts incubated with BPV-2 and donkey serum from a clinically normal donkey; S2-S4, wells containing bovine fetal fibroblasts incubated with BPV-2 and serum from three sarcoid-affected donkeys; V1-V3, wells containing bovine fetal fibroblasts incubated with BPV-2; C, wells containing bovine fetal fibroblasts incubated with neither

3.4. DISCUSSION

3.4.1. Immunoblot assay

The immunoblot assays described in this chapter have demonstrated the presence of antibodies reactive with disrupted BPV-2 virion and the L1 fusion protein of BPV-2 in the vast majority of sera from both sarcoid-affected and clinically normal donkeys. Detection rates against virion and L1 fusion protein were 86 and 96 per cent in sarcoid-affected and healthy animals combined. No antibody was demonstrable in the serum from a precolostrum donkey foal and as the donkey neonate receives its passive immunity from its dam's colostrum, not via the placenta, this foal may be considered to have been immunologically naive. Assay against dirupted virion demonstrated antibodies to a 55kDa antigen and a 65kDa antigen. The stronger signal was detectable at the 55kDa size and this corresponds to the L1 component of the major capsid protein. The 65kDa antigenic band is approximately the expected size of the L2 encoded capsid protein of BPV-2. Unfortunately, no assays using the L2 encoded fusion protein were carried out to confirm that the immune system of the donkeys recognised viral L2 encoded proteins, as suggested by the 65kDa apparent when disrupted virion was used as the target antigen. However, the fact that the control rabbit antisera only reacted with the 55kDa antigen may indicate a difference either in host response or between experimental and natural infection or that the 65kDa band is artefactual, possibly related to the cytokeratins of the bovine skin. More work, using the cloned L2 fusion protein would provide the additional information necessary to resolve this query.

The demonstration of antibody to the L1 fusion protein, in addition to the pre-incubation and adsorption data, suggest that the antibody reactive with the disrupted virion is indeed directed at the papillomaviral antigen and not at the cytokeratins that may have been present. The assays against the two components of the L1 fusion protein also indicate that epitopes are located on both the C

In view of the herd management at The Donkey Sanctuary, it was impossible to identify animals that had never been in contact with a sarcoid-affected donkey but it appears that presence or absence of antibody to papillomaviral antigen was unrelated to clinical status. The proportion of animals with antibody to the antigens used in the immunoassays is, at first consideration, surprisingly high. On purely clinical grounds from observations made in the human population, it is known that HPV infection occurs commonly in the general population and that immunosuppressed patients are at a high risk of developing HPV related disease (Benton, Shahidullah and Hunter, 1992). This occurs in patients with AIDS (Valle, 1987) and also in patients in receipt of a renal allograft, and who are consequently receiving immunosuppressive drugs: Barr, Benton, McLaren, Bunney, Smith, Blessing and Hunter (1989) reported a 77 per cent prevalence of viral warts in patients with a graft life of five to 22 years and it would appear that papillomaviruses are therefore widely encountered by the human population. The ubiquitous nature of the papillomavirus is also supported by unpublished data of Jarrett and O'Neil. These workers detected antibody to disrupted BPV-2, employing the methodology described in this chapter, in 70 to 80 per cent of calves that were screened for previous exposure to papillomavirus, prior to use in vaccination studies. The calves were purchased when only a few months old and it is probable that, had the animals been older at purchase, a higher proportion would have had detectable antibody to BPV-2. The donkeys sampled in the present study were of various ages but, with the exception of the foals, all were in excess of one year old and had been kept in large groups. If papillomaviral infection at The Donkey Sanctuary is as widespread as would appear to be the case in bovidae, then there is a high probability that older donkeys, managed under herd conditions, will have been exposed to viral antigen.

The conclusion based on the immunoblot results that there is a high prevalence of papillomavirus infection in the donkey is supported by serological

(1990) demonstrated antibody against the late proteins of HPV-6 in approximately 60 per cent of subjects, against HPV-16 in over 50 per cent of subjects and against HPV-18 in 25 per cent of subjects. Again, there was no statistically significant difference between adult patients, at a clinic for sexually transmitted diseases, and children and it appears that there are differences in the prevalence of infection of viral types. However, there are also differences between the results of different research groups. Whilst the findings of Li, Shah, Seth and Gilden (1987) were in agreement with those of Jenison *et al.* (1990), the work of Strike, Bonnez, Rose and Reichmann (1989) failed to demonstrate antibodies to HPV-6 L1 and L2 proteins in the sera of 38 patients with HPV-6 infections and in 22 uninfected controls. There may be several reasons for this discrepancy (e.g., epitope availability in the fusion protein construct, assay sensitivity) but it is evident that there are still drawbacks in the use of fusion proteins for serological assay (Galloway, 1990).

Regardless of the differences between different groups of workers, the single biggest deficit of this approach to seroepidemiology is the inability to detect antibodies directed against the conformational epitopes of the papillomaviruses. Jarrett *et al.* (1991) demonstrated that antisera from calves and rabbits injected with the fusion proteins used in the present study were reactive with both engineered and viral L1 proteins and, that reciprocally, antisera raised against disrupted virus was reactive with the fusion proteins. This suggests that the fusion proteins share epitopes with virus which are recognised by the host immune system. These findings support the theory that the antibodies detected in the immunoblot assays were directed against papillomaviral antigen. However, the work of Steele and Gallimore (1990) emphasises the need for caution when interpreting serological assays based on linear epitopes. These workers compared the results of serological assay by Western (immuno) blot, which utilises linear epitopes, to immunoprecipitation and ELISA, both of which utilise conformational

when disrupted virion was used as the antigen in an ELISA, the detection rate was only 9/83. In the present study, it may therefore be the case that the exceptionally high detection rates of 86 per cent and 96 per cent in the donkey are in fact under-estimates, as both assays utilised linear epitopes. In addition, the reduction in signal intensity by pre-adsorption of sera with BPV-2 virion and BPV-2 L1 fusion protein (but not by *E. coli* proteins nor beta-galactosidase) gives reasonable grounds for concluding that the antibody detected in the immunoblot assay was directed against papillomaviral antigen.

When the health status of the donkeys is considered, all donkeys except one (863) with sarcoids or a history of sarcoids were positive for antibodies against the antigen targets. It is difficult to explain why this one animal should be antibody negative but a number of possibilities may be considered: first, even though the assays were repeated, the serum came from a single sampling and consequently if the sample had been exposed to excessive heat the antibody may have been denatured; secondly, the diagnosis of sarcoid in this particular case may have been incorrect and the donkey was, in fact, healthy; thirdly an administrative error may have occurred ascribing the wrong animal number to the sample; lastly, the assays may be detecting a papillomaviral antigen which has nothing to do with the development of sarcoids. Of the healthy animals, excluding the pre-colostrum neonate, 13/13 were positive for antibody reactive with disrupted virion and 18/21 (81 per cent) were positive for antibody reactive with the L1 fusion protein. Although this difference is not significant and it should be remembered that the five of the animals proving negative for antibodies to the L1 were not assayed against the disrupted virion, a larger sample population and more accurate animal-contact histories would benefit the study.

In conclusion, the majority of donkeys assayed, whether healthy or sarcoid-affected, appear to have encountered a papillomaviral or papillomaviral-like antigen, but this does not appear to be related to clinical development of sarcoids.

presence of antibody to conformational epitopes, providing an antigen target similar to that encountered *in vivo;* further purification of the fusion proteins, including the removal of the beta-galactosidase would produce cleaner blots; assays against the L2 encoded fusion protein, or those encoded by the early ORFs, may produce more data of immunological and epidemiological significance. However, until the clinical disease can be definitively related to an aetiological agent it is debatable whether it is worth pursuing this line of investigation.

3.4.2. Neutralisation assay

No neutralising antibody to BPV-2 was detected in the donkey sera assayed in the present study. The failure of the donkey sera to prevent transformation of the bovine fetal fibroblasts indicates that the antibody demonstrated by Western blot does not neutralise the transforming activity of BPV-2 *in vitro.* Previous work carried out on immunity to BPV infection in equidae is difficult to interpret. Cook and Olson (1951) were able to produce sarcoma-like tumours in 10/11 horses by the inoculation of material derived from bovine papillomas. There was also no evidence of acquired immunity as the horses were susceptible to repeated exposure. This finding was in contrast to those of Segre *et al.* (1955) and Ragland and Spencer (1968) who reported that horses with BPV induced tumours produced serum antibodies capable of neutralising BPV.

Unfortunately, all of these studies were performed before the identification of the various bovine papillomaviral types and it is possible that mixed papillomaviral types were inoculated. Segre *et al.* (1955) also noted that there was a variation in the response of different animals to inoculation as well as variation in the pathogenicity of viral isolates from different sources. In addition, the neutralising properties of the test sera were assayed by a brief incubation with an unspecified amount of virus followed by inoculation into the skin of susceptible horses. These limitations raise the question of the role of host susceptibility, purity

animals in any assay is likely to provide a variety of responses. Indeed, one clinically normal test horse was observed to have a non-heat labile neutralisation factor in its serum (Segre *et al.,* 1955). Further cause for concern in Segre's study (1955) is the failure of calves inoculated with BPV to produce neutralising antibody to the virus, a finding which contradicts work done before and since (Post and Terpestra, 1947; Jarrett *et al.* 1990a).

Ragland and Spencer (1968) were unable to demonstrate the presence of neutralising antibody to BPV in the sera of horses with sarcoid tumours and when BPV was inoculated intradermally into noramal and saroid-affected horses, both groups of animals developed fibroblastic nodules as well as neutralising antibody to BPV. They concluded that this evidence failed to support the theory that equine sarcoid was caused by BPV.

The present study has shown no neutralising antibody to BPV-2, detectable *in vitro*, in the serum of sarcoid-affected or clinically normal donkeys at The Donkey Sanctuary. There are several interpretations of this finding: First, BPV-2 is not the aetiological agent of the donkey sarcoid; secondly, antibody to BPV-2 in the donkey sera is not directed against the neutralising epitope of BPV-2 and immunity to sarcoids is determined by other components of the immune system; thirdly, the antibody detected by immunoblotting is an artefact caused by the disruption and absence of the conformational epitopes. However, the work of Jarrett *et al.* (1991) and the reduction of the signal intensity by pre-adsorption of the sera with BPV-2 virion or BPV-2 L1 indicate that this is unlikely.

3.5. Summary

It seems most likely that there has been widespread exposure of the sarcoid-affected and clinically normal animals at The Donkey Sanctuary to a papillomaviral or a papillomaviral-like antigen. Antibodies which are reactive to this antigenic material do not neutralise the transforming activity of BPV-2 *in vitro*.

antigen also remains a matter for speculation. Although donkeys at The Sanctuary have no contact with cattle, previous exposure to infected bovidae or accomodation associated with cattle may have occurred and despite the fact that all animals wear fly-repellant tags, the climate at The Sanctuary in the south-west of England supports a large fly population. These matters will be discussed in more detail in Chapter VIII.

Seroepidemiology can provide much information about a disease, but investigations into the sarcoid still await the isolation of an aetiological agent. The demonstration of virions from naturally-occurring sarcoids has not been reported.

CHAPTER IV

ATTEMPTS TO ISOLATE AN AETIOLOGICAL AGENT: EXTRACTION OF PAPILLOMAVIRAL VIRIONS FROM SARCOID TUMOURS.

CHAPTER IV

ATTEMPTS TO ISOLATE AN AETIOLOGICAL AGENT: EXTRACTION OF PAPILLOMAVIRAL VIRIONS FROM SARCOID TUMOURS.

4.1. INTRODUCTION

In order to fulfill Koch's postulates it is necessary to identify a potential aetiological agent before attempts can be made to transmit the disease to another animal by inoculation of the isolated agent. There have been several attempts to isolate papillomavirus virions from sarcoid material, but none has been successful (Pfister, 1984; Gorman, 1985; Lancaster, *et al.*, 1977). This is in contrast to the equine papilloma which has been shown to contain papillomavirus particles (Fulton, Doanne and McPherson, 1970; O'Banion, Reichmann and Sundberg, 1986; Hamada, Oymada, Yoshikawa, Yoshikawa and Itakura, 1990a; 1990b). The retrovirus isolated from an equine sarcoid-derived cell line (England *et al.*, 1973; Cheevers *et al.*, 1982) has since been shown to be endogenous retrovirus present in normal equine cells (Cheevers *et al.*, 1986) and it now seems unlikely that the virus is of aetiological significance.

The literature contains many accounts for the extraction of papillomavirus from tumours or transformed cells in culture (for example, Crawford, 1965; Favre, Breitburd, Croissant and Orth, 1975; O'Banion *et al.*, 1986), most starting with large quantities of pooled tumours. The isolation and characterisation of different viral types from the various clinical manifestations of bovine papillomavirus infection and the pooling of different types of lesion may lead to the isolation of mixed virus types. Given the clinical diversity of sarcoid lesions, the extractions in

zonal and isopycnic purification. His conclusion was that the purity of virus isolated by the former method was of a sufficiently high standard and had the advantage of a high yield of intact virions. The following method is an account of his technique wherein the tumour homogenate is layered on to a preformed sucrose gradient and subjected to centrifugation, separating the particles at a rate determined by their density.

4.2. MATERIALS AND METHODS

4.2.1. Solutions

Tris buffer x10; 116.9g of sodium chloride, 24.2g Tris and 6.7g EDTA were dissolved in 1900ml distilled water and adjusted to pH 8.0 with concentrated hydrochloric acid. The volume was then made up to 2 litres with distilled water.

20% sucrose; 216.2g of sucrose were dissolved in 1 litre distilled water and the refractive index (RI) and density (D) ascertained to be 0.3638 and 1.0829g/cm^3, respectively.

10% sucrose; Equal volumes of the 20% sucrose solution and distilled water were mixed and the RI and D ascertained to be 1.3478 and 1.04 g/cm^3, respectively.

Caesium chloride (1.5g/cm^3); 6.7183g of caesium chloride were dissolved in 10ml of Tris buffer such that the RI was 1.3182.

4.2.2. Source of tumours

Sarcoid tumours were obtained from clinical cases referred for therapy to the Hospital at The Donkey Sanctuary. Sarcoids from two horses referred to the University of Glasgow Veterinary School were also examined for the presence of papillomavirus.

4.2.3. Extraction and purification of virions from sarcoids

4.2.3.1. Extraction from superficial scrapings

A superficial scraping was taken from the epidermis of some of the lesions. The scraping was resuspended in 500ul PBS (pH 7.4) and sonicated for 2 minutes before examination for papillomavirus particles by negative staining transmission electron microscopy.

4.2.3.2. Extraction from sarcoid tumours

All tumours were stored in PBS (pH 7.4) and glycerol (1:1) for a minimum period of 14 days in order to soften the tissue and facilitate homogenisation. The tumours were cut up finely with scissors and a scalpel blade before being weighed. The tissue was resuspended in Tris buffer containing 0.5 per cent sodium deoxycholate and 0.5 per cent Nonidet P40. The final concentration was adjusted to 10 per cent weight per volume. The suspension was then homogenised on ice for 20 minutes using a Silverson homogeniser. The homogenate was clarified on a bench top centrifuge at 2000*g* for 10 minutes and the supernatant was further clarified in a Sorvall OTD 50 ultracentrifuge at 10000*g* for 10 minutes at 4°C using an SW-41 rotor. Sucrose velocity gradients were made in SW-41 ultraclear centrifuge tubes (Beckman) by layering 5mls of 10 to 20 per cent sucrose solution on to a bed of caesium chloride solution of density 1.5g/cm^3. The clarified supernatant was layered carefully on to the gradients and centrifugation was carried out at 40000*g* for 30 minutes at 4°C. The gradients were examined for light scattering viral bands by direct illumination and the centrifugate was fractionated by piercing the bottom of the centrifuge tube with a 21 gauge needle and collecting five drops per fraction into separate tubes. The optical density of each fraction measured and the fractions dialysed against three changes of Tris buffer at 4°C, before repeating the rate zonal purification.

4.2.4. Examination by phosphotungstic acid negative staining transmission electron microscopy.

Each fraction was sonicated for 2 minutes and a 100ul aliquot placed on a parlodion grid for 1 minute. The excess sample was carefully blotted off and a 100ul aliquot of phophotungstic acid applied to the grid. The grids were once again blotted dry and examined for the presence of virions by transmission electron microscopy. In cases where there was a suspicion that virion-like particles were present, the corresponding fraction was subjected to ultracentrifugation at 30000*g* for 45 minutes and the resulting pellet resuspended in 200ul Tris buffer. The suspension was then examined for the presence of papillomaviral particles by the method described above.

4.3. RESULTS

All tumours were confirmed as sarcoids by microscopic examination of formalin-fixed paraffin-embedded tissue sections stained by haematoxylin and eosin. The clinical types of the lesions and the results of the extractions are presented in Table 23. Forty-nine fractions of tumour homogenate were examined for the presence of papillomaviral particles. Of the 49 fractions, 40 were extracted from ten tumours from eight donkeys, a further nine fractions from three tumours from two horses. Twenty-five of the 49 fractions were re-examined after ultracentrifugation to pellet material; 22 were from donkey tumours and three from horse tumours. Superficial scrapings were taken from 13 tumours from eight animals (seven donkeys, one horse). All four clinical types of tumour (Section 1.4.1.) were represented and two of the tumours were badly ulcerated with no intact epidermis. Of the 87 grids that were examined, only one grid contained visible particles resembling papillomavirus. The grid contained material from a fraction of an early type I or type IV sarcoid with an intact epidermis that was removed from a horse. The

by negative stain transmission electron microscopy and were approximately 55nm in diameter. Both intact and empty particles were present (Figure 14).

Table 23. Results of the negative staining electron microscopy of tumour homogenates and superficial scrapings.

Sample	Tumour	Tumour homogenate		Superficial Scraping	Total no.	No. positive
		No. of:				
Identity	Type	fractions	(pelleted)	No. of grids	of grids	for virion
Do1a	IIIU	5	(-)	1	6	0
Do1b	III	3	(3)	1	7	0
Do1c	I	-	(-)	1	1	0
Do2	I	1	(-)	1	2	0
Do3a	II	2	(2)	-	4	0
Do3b	I	6	(2)	-	8	0
Do4	II	10	(4)	1	11	0
Do5	II	3	(3)	-	6	0
Do6	IIU	3	(3)	1	7	0
Do7	I	3	(3)	-	6	0
Do8	I	4	(2)	-	6	0
Do9a	II	-	(-)	1	1	0
Do9b	IV	-	(-)	1	1	0
Do10a	I	-	(-)	1	1	0
Do10b	III	-	(-)	1	1	0
Do10c	II	-	(-)	1	1	0
Do11	II	-	(-)	1	1	0
Ho1a	I/IV	3	(-)	-	3	1
Ho1b	II	3	(-)	-	3	0
Ho2	II	3	(3)	1	7	0

Figure 14. Transmission electronmicrograph of papillomavirus-like particles extracted from an equine sarcoid, purified by rate zonal ultracentrifugation and negatively stained with poatassium phosphotungstate. The isolate contained both intact (closed arrow) and empty (open arrow) virions

4.4. DISCUSSION

The identification of papillomavirus-like particles in an extract from a sarcoid tumour has been described in this chapter. The particles were of a size, appearance and bouyant density similar to that of papillomaviruses although the presence of intact and empty capsids in the same fraction may indicate the disturbance of the gradient at fractionation. Although papillomaviral virions have been isolated from equine papillomas (Fulton, *et al.*, 1970; O'Banion *et al.*, 1986), this is the first report of a virion being isolated from an equine sarcoid. The tumour from which the virus was extracted in this study was a very early lesion (type I/IV) from a horse, which was only noticed when the animal was recumbant having been anaesthetised for the removal of a much larger periocular sarcoid. The present study is largely concerned with sarcoids in the donkey and the fact that the virion was found in a horse-derived lesion makes the observation somewhat incidental. However, the unique nature of this finding make it worthy of note.

In contrast to the horse lesion, the tumours which were removed from the donkeys at The Donkey Sanctuary were advanced lesions, generally several months old. The diagnosis of the disease in the donkey is hindered by the coarse, thick hair coat of the animal and by the fact that the animals at The Sanctuary are kept under herd management and consequently do not receive detailed clinical examinations frequently. It should be noted that the horse from which virus was isolated was privately owned, receiving daily grooming, and, even under this husbandry, the early sarcoid was not noticed. Whilst it was not possible to estimate the age of all the lesions included in this study, it is interesting to speculate that intact virus is only present in the very early pathogenesis of the disease, and that the well advanced donkey tumours were beyond the stage of viral production. Given that papillomaviruses require the terminal differentiation of the maturing epidermis to complete the vegetative cycle, it is not surprising that virions were not evident in ulcerated tumours or type II sarcoids, where there is little, if any,

and Lowy, 1980; Lowy *et al.,* 1980), but do not release progeny viral particles as the late genes are not transcribed in these cells (Heilman, Engel, Lowy and Howley, 1982).

The findings in this chapter are not sufficient to conclude that the papillomavirus is the cause of the equine sarcoid. In order to establish an agent as causative, Koch postulated that:

1) the agent must be found in all cases of the disease.

2) the agent must be isolated from a diseased host and grown in pure culture.

3) the agent must reproduce the disease when introduced to susceptible host.

4) the agent must be found to be present in the experimental host thus infected.

The isolation of virions from one lesion satisfies only partially the second postulate but the finding is further evidence implicating papillomaviruses in the aetiology of the disease. Although papillomaviruses have been detected in all types of cattle papillomas in this country (Jarrett, Campo, Moar, McNeil, Laird, O'Neil and Murphy, 1980), it has been shown that in the case of BPV-4 induced lesions neither virions nor viral nucleic acid were present in 68 of 70 lesions of the upper alimentary tract (Campo, Moar, Sartirana, Kennedy and Jarrett, 1985). It should also be noted that negative staining electron microscopy will detect virions at a minimum concentration of 10^8 particles/ml and it may be the case that virions were present in the tumours examined but at a lower concentration. Thus, the non-detection of virion in the majority of tumours examined in this chapter is not necessarily a reason for excluding the papillomavirus as an aetiological candidate.

The single isolate of virions described in this chapter provides a very small amount of material for future studies. Unfortunately, in order to study the biological properties of the virus particles in more detail and carry out transmission experiments a greater quantity of virus is required. The propagation of the virus remains a major constraint to research *in vitro* although the establishment of a keratinocyte line as a graft in nude mice (Sterling, Stanley

Development of this technique and others, such as organotypic (raft) culture systems (Meyers and Laimins, 1992) may eventually allow propagation of sufficient virus for transmission studies.

There were several reasons for suspending the virological investigations. First, all tumours from donkeys were negative for virus although this may have been due to the fact that the majority of the tumours from the donkeys were well advanced and had stopped producing virus. Secondly, the time consuming nature of the extraction of virions and, in particular, electron microscopic examination precluded the study being developed at the molecular level. Finally, the isolation of a papillomavirus from both papillomas (Fulton *et al.*, 1970; O'Banion *et al.*, 1986) and sarcoids does not distinguish between viral types or subtypes. It may be the case that the papillomavirus isolates of O'Banion *et al.* (1986) and the present study are identical, but such a distinction requires analysis at the level of the viral nucleic acid. It was for these reasons that the rest of the project was dedicated to investigating the molecular biology of the sarcoid in the donkey.

CHAPTER V

MOLECULAR INVESTIGATIONS: GENERAL MATERIALS AND METHODS

CHAPTER V

MOLECULAR INVESTIGATIONS: GENERAL MATERIALS AND METHODS

5.1. INTRODUCTION

This chapter summarises the materials and methods used routinely during the molecular component of the project. Other, specialised procedures, used infrequently, are described in the appropriate sections in subsequent chapters. The techniques and procedures employed during the investigation are essentially as described by Sambrook, Fritsch and Maniatis (1989) and Davis, Dibner and Battey (1986).

5.2. MATERIALS

5.2.1. Chemicals

All chemicals used in the molecular studies were of analaR, ultrapure or equivalent purity and were obtained from BDH Chemicals Ltd., Boehringer Mannheim Corporation, Gibco BRL, Pharmacia LKG and The Sigma Chemical Company except:

Ampicillin	Smithkline Beecham Ltd.
Ecoscint	International Diagnostics Inc.

5.2.2. Radiochemicals

[Alpha^{32}P]dCTP for labelling of DNA probes and [alpha^{35}S]dATP for DNA sequencing were obtained from Amersham International plc.

5.2.3. Equipment

Disposable plasticware	Scotlab, Gibco BRL.
Hybridisation membrane	Hybond N+, Amersham International plc.
Autoradiograph film	Kodak XAR Omat.
Filter paper	Whatman International Ltd.

5.2.4. Bacterial Strains

Cloning vectors were grown in *Eschericia coli* library efficiency DH5 alpha competent cells (BRL), genotype F-, endA1, hsdR17 (r_k^-, m_k^+), supE44, thi-1, lambda-, recA1, gyrA96, relA1, delta(argF-lacZYA)U169, phi80dlacZdeltaM15.

5.2.5. Cloning Vector

The pIC-20H modification of the pUC plasmid described by Marsh, Erfle and Wykes (1984) was utilised during cloning procedures. The vector was kindly provided by K.T. Smith.

5.2.6. Restriction endonucleases

All restriction endonucleases were obtained from Gibco BRL and were used with the appropriate reaction buffers.

5.2.7. Other enzymes

All enzymes were obtained from Gibco BRL, Boehringer Mannheim Corporation or The Sigma Chemical Corporation except:

Klenow fragment: Amersham International plc. in their Multiprime DNA labelling kit.

T7 DNA polymerase: United States Biochemical Corporation in their Sequenase^r version 2 DNA sequencing kit.

5.2.8. Antibiotics

Ampicillin: 1g sodium ampicillin dissolved in 10ml distilled water and sterilised by filtration.

5.2.9. Buffers and solutions

DNA gel loading buffer (6x): 0.25% bromophenol blue dissolved in a 40% solution (w/v) of sucrose in water.

EDTA (0.5M, pH 8.0): 186.1g disodium ethylene diamine tetracetate.$2H_20$ dissolved in 800ml distilled water with addition of approximately 20g NaOH pellets until pH 8.0 and adjustment of volume to 1l. Sterilised by autoclaving.

Phenol (Equilibrated, neutral): 250g of redistilled phenol was melted at 68^{o}C and extracted several times with equal volumes of 1M Tris HCl (pH 8.0) and then 0.1M Tris-HCl (pH 8.0) until the aqueous phase was at a pH less than 7.6. Stored in aliquots at -20^{o}C, protected from light.

Phenol (Equilibrated, acid): Same as above, except that 50mM sodium acetate (pH 4.0) was used in place of Tris-HCl (pH 8.0).

Plasmid-prep solution 1: 50 mM glucose, 10mM EDTA, 25mM Tris HCl (pH 8.0).

Plasmid-prep solution 2: (Freshly prepared) 0.2M NaOH, 1% SDS.

Plasmid-prep solution 3: 60ml 5M potassium acetate, 11ml glacial acetic acid, 28.5ml distilled water. This solution is 3M for potassium and 5M for acetate (pH 4.8).

Sodium acetate (3M): 408.1g sodium acetate.$3H_2O$ dissolved in 800 ml distilled water and pH adjusted to 5.2 by addition of glacial acetic acid. Volume adjusted to 1l and sterilised by autoclaving.

NaCl (5M): 292.2g NaCl dissolved in 800ml distilled water, adjusted to 1l and sterilised by autoclaving.

NaOH (10M): NaOH pellets disolved in 800ml distilled water adjusted to 1l and sterilised by autoclaving.

SDS (20%): 200g sodium dodecyl sulphate made up to 1l with distilled water and sterilised by filtering.

SSC (20x): 175.3g NaCl and 88.2g sodium citrate dissolved in 800ml distilled water, pH adjusted to pH 7.0 and volume adjusted to 1l. Sterilised by autoclaving.

TAE (50x): 242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA (pH 8.0) made up to 1l with distilled water.

TBE (10x): 108g Tris base, 55g boric acid, 40ml 0.5M EDTA (pH 8.0) made up to 1l with distilled water and adjusted to pH 8.3 and sterilised by autoclaving.

TE: 10mM Tris HCl, 1mM EDTA and adjusted to appropriate pH. Sterilised by autoclaving.

Tris (1M): 121g Tris base dissolved in 800ml distilled water and adjusted to appropriate pH with concentrated HCl. Volume adjusted to 1l and sterilised by autoclaving.

5.2.10. Bacterial growth media

L agar: L broth containing 1.5% agarose sterilised by autoclaving.

LB medium (L broth): 10g bacto-tryptone, 5g yeast extract and 10g NaCl made up to 1l with distilled water and pH adjusted to pH 7.3 with NaOH. Sterilised by autoclaving. Supplemented with ampicillin (50mg/ml), 2 per cent Xgal in DMF and 0.1M IPTG (filter sterilised)

SOC medium: 20g bacto-tryptone, 5g yeast extract, 0.5g NaCl dissolved in 950ml distilled water and 10ml of 250mM KCl solution added and pH adjusted to 7.0. Sterilised by autoclaving after volume adjusted to 1l. Just before use, 5ml sterile 2M magnesium chloride solution and 20ml sterile 1M glucose solution were added.

5.2.11. Hybridisation reagents

Denhardt's solution (100x): 2% bovine serum albumin (fraction V), 2% ficoll, 2% polyvinylpyrrolidone in distilled water. Sterilised filtering and stored at -20^{o}C.

Salmon testes DNA: 1g dessicated salmon testis DNA dissolved in 100ml distilled water by stirring for 5 hours at room temperature. DNA was sheared by passage through a 20 guage needle 10 times and then by boiling for 10 minutes. Stored in 1ml aliquots at -20^{o}C.

Hybridisation buffer (high stringency): For 1l of hybridisation buffer the following were combined: 500ml 50% formamide, 50ml 100x Denhardt's solution, 5ml 20% SDS, 50ml 1M Tris (pH 7.4), 20ml 0.5M EDTA, 150ml 20x SSC, 200ml 50% dextran sulphate, 625ul salmon sperm DNA (10mg/ml).

5.3. METHODS

5.3.1. Preparation of plasmid DNA.

In order to produce large amounts of cloned papillomaviral DNA for restriction endonuclease analysis or for the production of bovine papillomaviral DNA probes, the pAT153 plasmids, containing BPV-2, 4 and 5 genomes were amplified by the two methods outlined below.

5.3.1.1. Mini-preparation.

This was a modification of the alkaline lysis method of Birnboim and Doly (1979). A single bacterial colony containing the required plasmid was inoculated into 5ml of L broth containing ampicillin (100ug/ml) and incubated with shaking at 37^{o}C for 12 hours. The bacterial pellet was harvested after centrifugation at 2500*g* in a bench top centrifuge and any remaining supernatant aspirated. The pellet was resuspended in 100ul of Plasmid-prep solution 1 and transferred to a 1.5ml microcentrifuge tube, being allowed to stand at room temperature for 5 minutes. 200ul of ice cold freshly prepared Plasmid-prep solution 2 was added and the tube vortexed briefly and incubated on ice for 5 minutes. 150ul of Plasmid-prep solution 3 was then added and the tube incubated at 4^{o}C for a further 5 minutes. Cell debris and precipitated SDS were pelleted by centrifugation at 14000*g* in an eppendorf centrifuge for 5 minutes and the supernatant transferred to a fresh tube. An equal volume of 1:1 v/v equilibrated SS-phenol/chloroform/isoamyl alcohol was added and the mixture vortexed and centrifuged as before. If the DNA was to be used for nucleotide base sequence analysis, this phenol/chloroform step was replaced by the addition of an equal volume of acid phenol and separate chloroform extraction. In both cases, the upper aqueous phase was removed and one tenth volume 5M sodium chloride solution or 3M sodium acetate solution added followed by two volumes of absolute alcohol. After 5 minutes on dry ice the precipitated DNA was pelleted by centrifugation at 14000*g*. The supernatant was

pellet was dried at 37°C for 15 minutes and resuspended in 50ul of TE containing 50ug/ml DNAase-free pancreatic RNAase.

5.3.1.2 Large scale preparation

The use of the large scale preparation was largely superseded by the reliable miniprep method described above but in order to produce large amounts of bovine papillomaviral DNA probes several large scale preparations were performed in the early period of the project. The procedure was a modified version (Hettle, 1985) of the alkaline lysis described by Sambrook *et al.* (1989). A single bacterial colony containing the plasmid of interest was inoculated into 200ml L broth containing ampicillin (100ul/ml). The bacterial pellet was harvested by centrifugation in a 250ml polycarbonate flask at 4°C in a Beckman JS-7.5 rotor at 10000*g* for 15 minutes. The supernatant was discarded and the pellet resuspended in 10ml Plasmid-prep solution 1 and transferred to a polypropylene tube. Twenty ml of freshly prepared Plasmid-prep solution 2 were added and the contents of the tube mixed gently by inverting several times. After a 15 minute incubation, 15ml of ice-cold Plasmid-prep solution 3 was added and the tube shaken gently. The bacterial debris was then pelleted by centrifugation at 4000*g* at 4°C for 15 minutes and the supernatant filtered through several sterile tissues and 0.6 volumes of isopropanol added. The contents of the tube were mixed thoroughly, incubated at room temperature for 10 minutes and centrifuged at 5000*g* for 15 minutes in a Sorvall GS3 centrifuge. The pelleted nucleic acids were air dried, rinsed briefly in 70 per cent ethanol and resuspended in 3ml TE (pH 8.0).

5.3.2. Purification of closed circular DNA by equilibrium centrifugation in caesium chloride-ethidium bromide gradient.

The volume of the DNA solution containing the plasmid DNA was measured and 1g/ml of solid caesium chloride added. For every 10ml of the resulting solution,

clear pink solution from the centrifugate was transferred to Beckman Quick-Seal tubes and the remainder of the tubes filled with light paraffin oil. The gradients were then centrifuged at 40000*g* for 36 hours at 20°C in a Beckman Ti65 rotor after which the lower band visible in the body of the gradient was removed using a syringe and 21-guage needle. The ethidium bromide was then removed from this fraction of closed circular plasmid DNA by several extractions with 1-butanol saturated with water. The caesium chloride was removed by the addition of two volumes of water and the DNA precipitated by the addition of two volumes of ethanol. The DNA was recovered by centrifugation at 10000*g* for 15 minutes at 4°C in a benchtop microcentrifuge.

5.3.3. Extraction of Genomic DNA from tumour sections

Approximately 5g of freshly dissected tumour or snap frozen tissue were dissected finely with scalpel and forceps. The tissue was then suspended in approximately 10ml PBS (pH 7.4) to which was added 1ml of 10 per cent SDS and 11mg proteinase K powder. The mixture was then incubated at 37°C for 4 hours after which time a further 11mg proteinase K was added. The mixture was incubated for a further 12 hours at 37°C with gentle agitation. The solution was clarified by brief centrifugation on a benchtop centrifuge, the supernatant collected and one tenth volume of 3M sodium acetate solution added. The aqueous phase was then extracted twice with SS-phenol:chloroform: isoamyl alcohol (10:10:1) and once with diethyl ether. The nucleic acids were precipitated by the addition of 2.5 volumes of absolute ethanol and removed by spooling out on a sealed pasteur pipette. Following a brief wash in 70 per cent ethanol, the nucleic acids were resuspended in 3ml TE buffer containing 50ug/ml DNAase-free pancreatic RNAase to remove any residual RNA.

5.3.4. Agarose gel electrophoresis

1x TAE. The running buffer was either 1x TBE or 1x TAE depending upon which of the buffers had been used to cast the gel. Each sample of DNA was mixed with a 0.2 volumes of gel loading buffer. If genomic DNA was being electrophoresed following restriction endonuclease digestion, the sample was heated to 65°C for 10 minutes in order to to disrupt hydrogen bonds between molecules with compatible cohesive termini. Electrophoresis was carried out at approximately 5V/cm, usually overnight. Following electrophoresis the DNA was stained by the addition of 0.5ug/ml ethidium bromide to the gel running buffer for 45 minutes. The gel was destained by washing in distilled water for 45 minutes. The stained DNA was visualised by viewing the gel under long wave ultraviolet light on a transilluminator. (Vilbour Lourmat, 312nm wavelength output). The gel was photographed using Polaroid type 57 high-speed film on the Polaroid MP-4 camera system. On all gels the size markers used were the *Hin*dIII digest of bacteriophage lambda genomic DNA. When a rapid result was required a minigel apparatus was used to run the agarose gel, allowing a potential of 20V/cm to be applied for 30 to 40 minutes. Ethidium bromide was not routinely added to the gel at casting as the relative mobilities of supercoiled, circular and linear DNA are altered by the intercalation of the dye with the DNA molecules.

5.3.5. Extraction of DNA from low melting point agarose gels

Preparation of the LMP agarose gels and DNA samples was carried out as described in section 5.3.4. The DNA fragment of interest was visualised by brief exposure to ultraviolet light and the section of agarose removed using a scalpel and transferred to a microcentrifuge tube. The agarose was then melted by incubation at 65°C for 10 minutes and two volumes of TBE added. The diluted sample was allowed to cool in a waterbath at 37°C and an equal volume of equilibrated phenol was then added. The mixture was vortexed and the aqueous phase collected after centrifugation at 14000*g* for 5 minutes. The sample was

5.3.6. Southern blotting

The alkaline transfer modification of the capillary transfer described by Southern (1975) was used for all blotting procedures. This technique allows rapid transfer of nucleic acids as the alkaline transfer medium neutralises, hydrolyses and finally covalently binds the DNA to the nylon membrane. It has the disadvantage of having higher background than other methods during subsequent hybridisation experiments. Following agarose gel electrophoresis, staining and photography, the gel was washed gently in 0.25M HCl for 20 minutes in order to depurinate the DNA . The gel was then transferred briefly to distilled water before being placed on a wick of 3MM Whatman paper on a solid support. The wick had been previously soaked in 0.4M NaOH. A precut piece of positively charged nylon membrane the same size as the gel was then laid on top of the gel and was covered in turn by a layer of 3MM Whatman paper. Paper towels were then stacked on top of the assembly and light pressure applied with a 1kg weight. Approximately 500ml 0.4M NaOH was then added to the reservoir below the wick and the capillary transfer of the nucleic acids allowed to proceed for a maximum time of 5 hours. The paper towels were changed after 2 hours. The apparatus was dismantled and the nylon membrane washed briefly in 2xSSC and then air dried.

5.3.7. Pre-hybridisation

The filter was laid flat in a heat sealable plastic bag and the bag double sealed on three sides. A 625ul aliquot of salmon sperm DNA was heated to 100^{o}C for 5 minutes, chilled on ice for a further 5 minutes and then added to 25ml of hybridisation buffer preheated to 37^{o}C. The mixture was then added to the plastic bag and, after excluding all air bubbles, was double-sealed along the remaining edge. The bag was then placed in a plastic sandwich box and placed in a rocking waterbath at 42^{o}C. Prehybridisation carried out for 4 to 16 hours.

5.3.8. Hybridisation

The prelabelled probe was thawed and the required volume aliquoted into a screw-top eppendorf. The volume was calculated by the following formula:

$$\text{Volume} = 25 \times [(N_0 \times e^{-kt})/10^6]$$

where N_0 is the counts per minute of 1ul of the probe

k is a constant

t is the number of days since the cpm were measured.

This formula gives the volume of probe required for addition to 25ml hybridisation buffer sufficient to produce an activity of $1x10^6$ cpm/ml. The probe was then heated to 100°C for 5 minutes, chilled on ice for a further 5 minutes and injected into the bag containing the hybridisation buffer using a 1ml syringe and a 1 inch 21 guage needle. The hole created by the needle was double sealed using a bag sealer. Hybridisation was carried out for 16 hours at 42°C.

5.3.9. Washing the filters

Although the equation regarding stringency of hybridisation (Meinkoth and Wahl, 1984) which states that,

$$T_m = 81.5^oC + 16.6\log M + 0.41(\%G+C) - 500/L - 0.61(\%\text{formamide})$$

Where, T_m = melting temperature

M = molarity of the hybridisation solution

L = probe length

was taken into consideration, it was not found to be particularly useful because random priming produces probes of varying lengths and the G/C content of the target and the degree of homology was unknown at the outset of the project. Two different stringencies were used for the washing of the membranes following hybridisation. In a low stringency wash, the membranes were washed twice for 20 minutes at 42°C in 2x SSC, 0.1 per cent SDS and twice for 30 minutes at 42°C in

washed twice for 20 minutes at 42°C in 2x SSC, 0.1 per cent SDS and twice for 30 minutes at 65°C in 0.5x SSC, 0.1 per cent SDS. In both cases, the blots were then monitored for radioactive emissions with hand-held Geiger-counter and the washes repeated until the level of activity was less than 2cpm. The excess fluid was then blotted off the membranes which were then wrapped in Saran wrap before autoradiography.

5.3.10. Preparation and purification of synthetic oligonucleotides

Oligonucleotides 15 nucleotides in length were synthesised using an Applied Biosystems 381A DNA synthesiser. The 5' methoxy trityl protecting group was removed after the incorporation of the last (5') nucleotide. The oligonucleotides were eluted from the synthesis column by the gradual introduction of 2ml 30 per cent ammonia solution, 0.25ml ammonia solution being introduced to the cartridge every 30 minutes, passing from one 2ml syringe to another. After elution was complete the solution was transferred to a screw top microcentrifuge tube and incubated at 55°C overnight in order to remove the side-chain protecting groups. The oligonucleotide was precipitated as described for other types of DNA and quantified by spectrophotometry using the formula:

$O.D._{260}$ x Dilution factor x 20 = DNA concentration (ug/ml).

5.3.11. Restriction endonuclease digestions

A number of different restriction endonucleases (REs) were used in the preparation and characterisation of the cloned and genomic DNA. In the case of the plasmid DNA, 1 to 2ug of plasmid DNA were digested in a total reaction volume of 20ul. When digesting genomic DNA, 5 to 10ug was incubated with the RE in a total volume of 50ul. Restriction enzymes were used at a concentration of 3 units per 1ug of substrate DNA. RE digestions were carried out at 37°C for 2

5.3.12. ^{32}P radio-labelling of dsDNA.

This was carried out with the Multiprime labelling kit obtained from Amersham International plc. The method was first described by Feinberg and Vogelstein (1983) utilising random sequence hexanucleotides to prime DNA synthesis from a denatured DNA template. Synthesis of the DNA was carried out employing [alpha^{32}P]dCTP and unlabelled dATP, dGTP, dTTP. The Klenow fragment of *E. coli* DNA polymerase was used to catalyse the reaction and as this enzyme lacks 5' to 3' exonuclease activity, the product was not degraded once synthesised. Twenty-five ng of DNA in 25ul water was heated to 98^{O}C for 5 minutes and placed on ice. Ten ul of Multiprime buffer containing the unlabelled nucleotides, in a buffer of Tris HCl (pH 7.8) magnesium chloride and 2-betamercaptoethanol, were added as well as 5ul of primer, an aqueous solution of random hexanucleotides containing nuclease free bovine serum. Eight ul of [alpha^{32}P]dCTP (800Ci/mmol, 10uCi/ul), observing the normal precautions and working in a dedicated area were added followed by 2 units of Klenow fragment and the reaction incubated at room temperature overnight. The unincorporated label was removed from the labelled oligonucleotides by chromatography through a 15cm column of Sephadex G-50. This procedure provided approximately 500ul of probe of activity 1.0 - 5.0 x 10^{5}cpm/ul. The precise activity was measured by adding 2ul of probe to 5ml Ecoscint and measuring the activity on a Beckman liquid scintillation counter. The amount of solution required for any one hybridisation was calculated as outlined in section 5.3.8. Probes were heated to 98^{O}C and chilled on ice before being used in hybridisation procedures.

CHAPTER VI

DETECTION, CLONING AND CHARACTERISATION OF PAPILLOMAVIRAL DNA PRESENT IN SARCOID TUMOURS

CHAPTER VI

DETECTION, CLONING AND CHARACTERISATION OF PAPILLOMAVIRAL DNA PRESENT IN SARCOID TUMOURS

6.1. INTRODUCTION

Despite the fact that there have been no previous reports of the isolation of papillomavirus virions from equine sarcoids, several groups have detected papillomaviral DNA in the tumours (Lancaster *et al.*, 1977; Lancaster *et al.*, 1979; Amtmann *et al.*, 1980; Lancaster, 1981; Trenfield *et al.*, 1985; Angelos, 1990; Angelos *et al.*, 1991). The majority of these studies have been conducted on the sarcoids of the horse although there is one report of papillomaviral DNA in a sarcoid tumour from a donkey (Amtmann *et al.*, 1980). The characterisation of the DNA detected in the tumours has been carried out to an elementary level by restriction endonuclease digestion of tumour genomic DNA, but there has been no report of the molecular cloning of any of the papillomaviral elements. The evidence from both liquid phase and Southern blot/hybridisation experiments points to the fact that the papillomaviral genomes involved are very closely related to BPV-1 or 2. Australian workers (Trenfield *et al.*, 1985) isolated DNA homologous at low stringency to BPV-1 and 2 but demonstrated a variety of restriction fragment patterns, whilst Angelos (1990) demonstrated BPV-1-like and BPV-2-like DNA in sarcoids from Switzerland and the United States. In contrast to the work on the sarcoid, O'Banion *et al.* (1986) described the cloning and characterisation of a papillomaviral genome extracted from the lesions of a horse with juvenile contagious papillomatosis, a disease clinically and pathologically

humans and cattle.

Prior to the advent of the polymerase chain reaction (Chapter VII) the molecular cloning of papillomaviral DNA was pre-requisite to the complete analysis of the viral genome by restriction endonuclease cleavage and nucleotide base sequence determinations. This chapter describes three aspects of the molecular biological investigations into sarcoids in the donkey: The detection of papillomaviral DNA in the genomic DNA extracted from donkey sarcoids and demonstration of a variety of restriction endonuclease cleavage patterns; the molecular cloning of a papillomaviral element into plasmid pIC-20H and restriction endonuclease analysis of the clone; the determination of the nucleotide base sequence of two regions of the cloned papillomaviral DNA.

6.2. MATERIALS AND METHODS

6.2.1. Probing of sarcoid derived DNA for the presence of papillomaviral DNA

The genomic DNA was extracted from the snap-frozen sarcoid tumours as described in Section 5.3.3.. Ten ug of this DNA was then digested with *Hin*dIII, *Bam*HI and *Kpn*I restriction endonucleases as described previously (Section 5.3.11.). Agarose gel electrophoresis, photography, Southern blotting, hybridisation to full-length molecularly cloned BPV-2, BPV-5 and BPV-4 genomes and autoradiography were performed exactly as described in the previous chapter.

6.2.2. Molecular cloning of a papillomaviral element extracted from sarcoid Do1b

6.2.2.1. Dephosphorylation of the plasmid vector

Five ug of plasmid pIC-20H were digested with *Hin*dIII in 20ul at 37°C for 60 minutes, linearising the DNA at the unique *Hin*dIII site. In order to prevent religation of the compatible ends, the 5' phosphate was removed by incubation with 1ul alkaline phosphatase (3U/ul) in 24ul water and 5ul alkaline phosphatase buffer (0.5M Tris (pH 9.0), 10mM magnesium chloride, 1mM zinc chloride) at

6.2.2.2. Isolation and ligation of insert

A *Hin*dIII digest of 5ug of the sarcoid-derived genomic DNA from sarcoid Do1b of donkey 3590 was electrophoresed through a 0.8 per cent LMP agarose gel. The fragments of approximately 8kbp in size were cut from the gel after visualistion by staining with ethidium bromide. The DNA was recovered from the LMP gel as described (Section 5.3.5.), precipitated with ethanol and resuspended in TE buffer (pH 8.0). The concentration of the DNA solution was estimated by measuring the $OD_{260/280}$ ratio and adjusted to 150ng/ul. Two ul (300ng) of this DNA was then combined with 1ul (100ng/ul) of the prepared plasmid pIC-20H, 1ul T4 DNA ligase, 2ul 10X ligase buffer and 14ul water. The ligation reaction was incubated at 14^{o}C overnight and the success of the ligation checked by agarose gel electrophoresis.

6.2.2.3. Transfection of competent cells

Library efficiency DH5alpha cells prepared by BRL's modification of Hanahan's procedure were used to produce a library of *Hin*dIII digest of sarcoid derived DNA. The phi 80*lac*ZdeltaM15 marker provides alpha-complementation of the beta-galactosidase gene from pUC derived vectors and was used to produce blue/white screening of colonies on bacterial plates containing X-gal. A 100ul volume of competent cells were aliquoted from a purchased stock into a chilled polypropylene tube (Falcon 2059). The DNA from the ligation reaction was diluted fivefold in TE (pH 7.5) and 1-5ul of the dilution (1-10ng) were added to the cells which were then incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42^{o}C and returned to ice for a further 2 minutes. After the addition of 0.9ml SOC at room temperature the cells were incubated at 37^{o}C and shaken at 225rpm for 1 hour. Approximately 150ul of the reaction mixture was spread onto L broth/agar plates containing 50mg/ml ampicillin, 2 per cent Xgal in DMF and 0.1M IPTG. The plates were incubated at 37^{o}C overnight in an inverted

6.2.2.4. Screening of transfected bacterial colonies

The efficiency of the transfection was assessed by the number white colonies produced after overnight incubation, demonstrating that the plasmids containing the inserts were failing to complement the alpha-complementing *E. coli*. However, in order to identify the plasmids containing papillomaviral DNA, the colonies were screened by hybridisation to molecularly cloned BPV-2 genome, in a manner similar to that outlined in Sections 5.3.7. and 5.3.8.. A circular piece of dry nitrocellulose was laid on top of the colonies and marked eccentrically with pinholes to facilitate relocation after hybridisation. When wetted, the filter was then floated on a solution of 0.5M sodium hydroxide, colony side up, for 5 minutes followed by flotation on a solution of 1.5M sodium chloride with 0.5M Tris (pH 7.4). Finally, the filter was laid gently on a solution of 1.5M sodium chloride and 2x SSC for 5 minutes before drying at 80^{o}C under vacuum. The pre-hybridisation and hybridisation procedures were then identical to those used for any other filter and, after autoradiography, the positive colonies were identified by placing the bacterial plate over the radiographic film. Positive colonies were picked on a sterile loop and grown in L broth as described in Section 5.3.1. prior to RE analysis or nucleotide base sequence determination. One ug aliquots of the cloned papillomaviral DNA were digested with *Hin*dIII, *Bam*HI, *Eco*RI, *Kpn*I, *Sma*I, *Sst*I and *Xba*I in order to characterise the cloned genome. Agarose gel electrophoresis, Southern transfer and hybridisation to molecularly cloned BPV-2 were performed as described previously in Chapter V. The genomic organisation of the clone was then compared to that of BPV-1 and BPV-2 (Lancaster, 1979; Engel, Heilman and Howley, 1983)

6.2.3. Nucleotide sequence analysis of molecularly cloned papillomaviral element

6.2.3.1. Introduction

All nucleotide sequence analysis was performed on alkali denatured double-

obtained from such templates, obviating the need for production of single stranded DNA templates. In order to obtain good sequence data, the DNA templates must be of high quality. The first sequencing experiments in this project were performed on plasmid DNA purified by the equilibrium centrifugation in caesium chloride/ethidium bromide gradients. The source of template DNA was replaced by that purified by acid-phenol minipreparation of plasmid DNA as it was found that the results from this source were of equal or better quality than that from the caesium chloride gradient purification. In contrast to the acid equilibrated phenol, Tris equilibrated phenol was found to be unsuitable for the purification of plasmid for use in sequencing reactions. Of the available modern T7 DNA polymerases, the genetically engineered Sequenase Version 2.0 (US Biochemical Corporation) was used, having a lower 3' to 5' exonuclease activity and higher processivity than the native enzyme and, in order to use the high processivity to best advantage, the sequencing reaction was divided into two stages. In the first, low concentrations of the dNTPs and a low incubation temperature (room temperature) ensured the efficient incorporation of the single radiolabelled dNTP at the same time as limiting DNA synthesis. In the second stage, the mixture was divided into the four standard reactions each containing high concentrations of one of the four dNTPs and a single dideoxy NTP in order to terminate the polymerisation. The Sequenase Version 2.0 sequencing kit (US Biochemical Corporation) and manufacturers protocol were used and the cloning vector was pIC-20H as described in Sections 5.2.5.. Two primers were used; one was the Universal M13 primer, the other a custom-synthesised 15mer selected from the E5 ORF of BPV-1.

6.2.3.2. Production of single stranded template.

Three to 5ug of pIC-20H DNA containing the papillomaviral element from sarcoid Do1b of donkey 3590 and purified by acid-phenol minipreparation were incubated with 0.2M NaOH and 2mM EDTA for 30 minutes at 37°C. The mixture was

resuspended in 7ul of distilled water.

6.2.3.3. Annealing template and primer.

A 10ul reaction volume, comprising 1ul universal M13 primer or custom-synthesised primer, 2ul reaction buffer and 7ul (1ug) of DNA, was heated to 65°C for 2 minutes and then cooled slowly to room temperature allowing the primer to anneal to the template. The annealed template and primer were placed on ice after the temperature had fallen below 30°C.

6.2.3.4. Labelling reaction

The labelling mix supplied with the kit containing dGTP, dCTP and dTTP was diluted five-fold with distilled water and the Sequenase enzyme diluted seven-fold with ice-cold enzyme dilution buffer. The annealed template-primer mix was then placed on ice and 1ul 0.1M DTT, 2.0ul diluted labelling mix 0.5ul [alpha-^{35}S]dATP and 2.0ul diluted Sequenase enzyme added and the mixture incubated at room temperature for 5 minutes.

6.2.3.5. Termination reaction

Four 500ul tubes were labelled G, A, T and C and 2.5ul of the appropriate termination mix added to each tube. The ddNTPs contained in these mixes lacked the 3'-OH group necessary for DNA chain elongation and thus terminated the reaction. The tubes were heated to 37°C for 1 minute after which time 3.5ul of the labelling reaction mixture was transferred to the corresponding tube. The mixtures were incubated for a further 5 minutes and then 4ul of stop solution added to each tube. The reactions were then stored at -20°C for up to one week.

6.2.3.6. Denaturing gel electrophoresis

The two glass plates of a customised sequencing rig were cleaned with alcohol and then coated on the inner surfaces with 5 per cent dichloromethyl silane in

sides with waterproof plastic tape. The denaturing PAGE gel mix containing 23ml 30 per cent acrylamide, 50g urea, 10ml 10xTBE, distilled water to 100ml, 333ul 10 per cent ammonium persulphate and 83ul TEMED was then introduced between the plates using a 50ml syringe and avoiding the introduction of air bubbles. A comb was then inserted and the gel left to polymerise for 4 to 12 hours. Once polymerised, the tape was removed from the bottom edge of the gel assembly, which was then clamped in position between the upper and lower buffer chambers of the electrophoresis cell. Sufficient 0.5x TBE was added to ensure that the electrodes were covered and the comb was removed carefully from the gel. The wells were washed out gently by syringing buffer into them in order to remove any unpolymerised acrylamide and bubbles trapped on the bottom edge of the gel assembly were dislodged. The apparatus was sealed in a safety cabinet, connected to a high voltage power supply and the gel was run at appoximately 50W constant power. This setting depended upon the individual gel mixture and buffer strength but at no time was the current allowed to exceed 25mA because the heat generated at currents in excess of this cracked the glass plates. The gel was pre-run for approximately 30 minutes before flushing the wells and loading the 2ul of each sequencing reaction mixture which had been heated to 95^{o}C immediately prior to electrophoresis. The gel was run for two to six hours or until the xylene cyanol had migrated approximately 60cm.

Following electrophoresis the gel assembly was dismantled and the gel soaked in 2l of ten per cent acetic acid and 12 per cent methanol in order to remove the urea. The gel was transferred to a sheet of 3MM Whatman paper, covered in cling film and dried under vacuum at 80^{o}C for 2 hours on a Biorad 853 gel drier. The dried gel was exposed to Kodak XAR-Omat film at room temperature for between 24 and 48 hours. The sequence was analysed and compared to the published sequences of characterised papillomaviruses using the BESTFIT programme of the GCG sequence analysis software (University of

6.3. RESULTS

6.3.1. Detection and partial characterisation of papillomaviral DNA

Eighteen sarcoids, one melanoma, one mass of granulation tissue and one sample of normal donkey skin were assayed for the presence of papillomaviral DNA by Southern blot and hybridisation of *Hin*dIII digests of the extracted DNA to molecularly cloned full-length genomes of BPV-2, 4 and 5. The identity of each tissue sample was confirmed by microscopic examination of formalin-fixed paraffin-embedded tissue sections stained with haematoxylin and eosin and the results are summarised in Table 24, together with the results of the further analysis of 15 of the sarcoids. All of the sarcoids contained DNA which hybridised to molecularly cloned full-length BPV-2, under stringent conditions, and was generally present in three forms; linear, open circle and supercoiled DNA (Figure 15). The DNA in the sarcoid samples did not hybridise to either BPV-4 or BPV-5 DNA probes at high or low stringency nor was there any DNA which hybridised to BPV-2 DNA in that extracted from the melanoma, the granulation tissue or the normal donkey skin.

Fifteen of the DNA samples extracted from the sarcoid tumours were subjected to further restriction endonuclease analysis using *Bam*HI and *Kpn*I REs and the results of these digests are also summarised in Table 24. Three different RE cleavage patterns were obtained: Three of the tumours contained papillomaviral DNA identical in size and RE cleavage pattern to BPV-1; three tumours contained DNA identical in size and RE cleavage pattern to BPV-2; nine tumours contained DNA identical in size to BPV-1 and 2 but had a RE cleavage pattern similar to both, but identical to neither, BPV-1 or 2. This third isolate was approximately 8kbp in length with single *Hin*dIII and *Bam*HI sites. The isolate also had two *Kpn*I sites yielding two fragments approximately 4250 and 3750bp in size. There did not appear to be any correlation between the type of papillomaviral DNA and the clinical type and anatomical location of the lesion, nor with the sex

Table 24. Summary of the screening of donkey sarcoids for papillomaviral DNA after restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting and hybridisation to full-length molecularly cloned BPV-2 genome.

Donkey	Sex	Sarcoid No.	Sarcoid Type	Anatomical Site	RE digest : No. of fragments			DNA Identity
					*Hin*dIII	*Bam*HI	*Kpn*I	
3590	Male	Do1a	IIU	Penis	1	1	1	BPV-1
514	Female	Do15	IIU	Abdomen	1	1	1	BPV-1
2077	Male	Do6	IIU	Abdomen	1	1	1	BPV-1
3075	Female	Do16	III	Nostril	1	2	2 equal	BPV-2
3037	Male	Do17	III	Brisket	1	2	2 equal	BPV-2
2515	Male	Do18	I	Periocular	1	2	2 equal	BPV-2
3590	Male	Do1d	II	Prepuce	1	1	2	Novel
3590	Male	Do1b	III	Stifle	1	1	2	Novel
2087	Male	Do12	II	Penis	1	1	2	Novel
876	Female	Do13	II	Abdomen	1	1	2	Novel
R2	Male	Do3b	I	Lips (head)	1	1	2	Novel
R2	Male	Do3a	II	Prepuce	1	1	2	Novel
3459	Male	Do9a	II	Prepuce	1	1	2	Novel
2529	Male	Do14a	III	Prepuce	1	1	2	Novel
2529	Male	Do14b	II	Abdomen	1	1	2	Novel
2428	Female	Do15	IIIU	Periocular	1	N/A	N/A	PV
3675	Male	Do16	I	Lips(head)	1	N/A	N/A	PV
1708	Female	Do10c	II	Chin	1	N/A	N/A	PV
2529	Male	NS2	granulation tissue	Abdomen	0	N/A	N/A	None
3590	Male	NS1	normal skin	Scrotum	0	N/A	N/A	None
3472	Female	Do5	melanoma	Vulva	0	N/A	N/A	None

RE; restriction endonuclease
U ; Ulcerated
PV; papillomaviral DNA
N/A; Not assayed

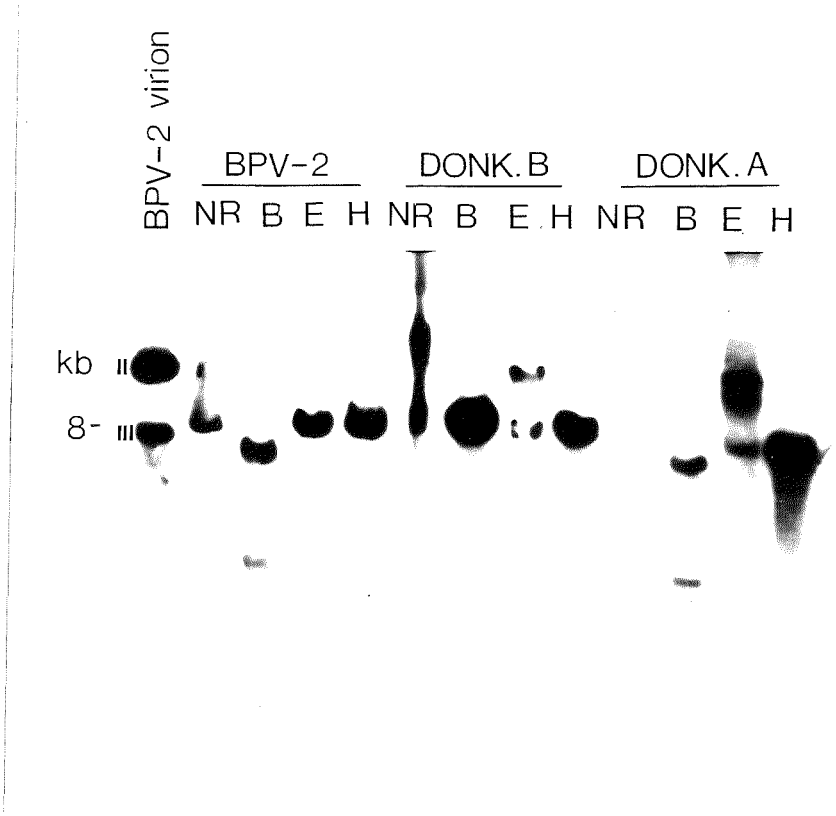


Figure 15. Autoradiograph after Southern blot and hybridisation showing viral DNA detected in sarcoid tumours from two donkeys and comparison to cloned BPV-2 genome following restriction endonuclease digestion. NR, undigested DNA; B, DNA digested with *Bam*HI, E, DNA digested with *Eco*RI, H, DNA digested with *Hin*dIII. Hybridisation was to molecularly cloned BPV-2 DNA. *Eco*RI was found to produce inconsistent results when used to digest high molecular weight DNA and was only used in pilot screening hybridisations being replaced by *Kpn*I. In the majority of samples relaxed circle (II) and linear (III) forms of the viral DNA were present. The supercoiled (I) form is not evident on this autoradiograph.

6.3.2. Molecular cloning and characteristion of a papillomaviral element from tumour Dolb.

One of the novel isolates, identified by the RE digests of DNA from sarcoid Dolb, was molecularly cloned into the unique *Hin*dIII site in the vector pIC-20H as described above. The RE digests using two REs were carried out on mini-preparations of the resulting plasmid DNA, assigned $pDoPV_{IC\text{-}20H}$. The plasmid was digested with *Hin*dIII yielding a linearised vector, approximately 2.7kbp in size, and the linearised insert, DoPV, approximately 8kbp in size (Figure 16). The DoPV insert was then analysed further using *Bam*HI, *Eco*RI, *Kpn*I, *Sma*I, *Sst*I and *Xba*I REs. The nature of the DNA visualised after agarose gel electrophoresis was confirmed by Southern blotting and hybridisation to full-length molecularly cloned BPV-2 genome. The BPV-2 probe did not hybridise to the plasmid DNA as there was no 2.7kbp band visible following autoradiography. The results of the RE digests are summarised in Tables 25 and 26 and in Figure 16. It appeared that the DoPV shared RE sites with both BPV-1 and BPV-2: Like both BPV-1 and 2, DoPV had a single *Hin*dIII site; like BPV-1, DoPV had single *Bam*HI, *Eco*RI and *Sma*I sites and lacked an *Sst*I site; like BPV-2, DoPV had two *Kpn*I sites but the fragments were of different sizes (4250bp and 3750bp); unlike BPV-1 or 2, DoPV had no *Xba*I site.

6.3.3. Nucleotide base sequence analysis of DoPV

The results of the nucleotide base sequence analysis indicated that the DoPV was practically identical to BPV-1 in both the L1 ORF and the E5 ORF (Figure 17). Comparison of the DoPV genome with BPV-1 by BESTFIT local homology (GCG sequence analysis software) indicated that in the L1, the per cent identity, or homology, was 95.6 per cent with four gaps and, in the E5 ORF, the homology was 98.0 per cent, with no gaps. A similar comparison of DoPV with BPV-2 revealed a 83.9 and 89.8 per cent homology in the L1 and E5 sequences of the two viruses,

and 89.8 per cent homology in the L1 and E5, respectively, thus demonstrating that the DoPV sequences had greater homology with BPV-1 than does BPV-2.

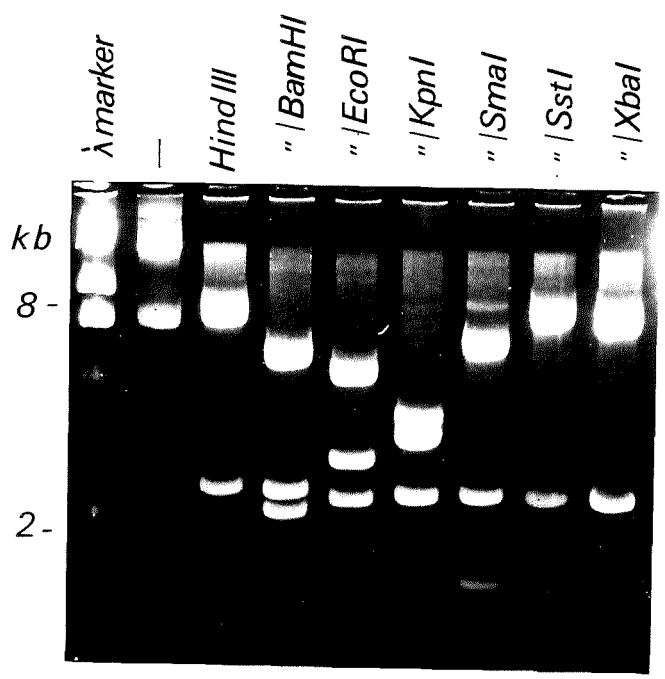


Figure 16. Agarose gel after staining with ethidium bromide solution and transillumination with UV light (320nm) showing the results of the restriction endonuclease digestion of the cloned papillomaviral element, $pDoV_{IC\text{-}20H}$, after electrophoresis. The lanes contained 1ug of the cloned DNA digested with *Hin*dIII alone or *Hin*dIII followed by the restriction endonuclease ascribed. The lane labelled "-- " contained undigested $pDoV_{IC\text{-}20H}$. The molecular weight marker used was a *Hin*dIII digest of lamda bacteriophage DNA. The arrow marks the plasmid vector DNA. A fourth

Table 25. Characterisation of the papillomaviral element (DoPV), isolated from sarcoid Dolb, by a single restriction endonuclease digestion, showing the number of cleavage sites and approximate fragment sizes (bp) with the corresponding results from the digestion of BPV-1 and BPV-2 genomes.

RE digest	DoPV		BPV-1		BPV-2	
	No. of sites	Fragment sizes	No. of sites	Fragment sizes	No. of sites	Fragment sizes
*Hin*dIII	1	8000	1	7945	1	7937
*Bam*HI	1	8000	1	7945	3	5879, 2031, 27
*Eco*RI	1	8000	1	7945	0	-
*Kpn*I	2	4250, 3750	1	7945	2	3998, 3939
*Sma*I	1	8000	1	7945	0	-
*Sst*I	0	-	0	-	1	7937
*Xba*I	0	-	1	7945	1	7937

Table 26. Characterisation of the papillomaviral element (DoPV), isolated from sarcoid Dolb, by double restriction endonuclease digestion showing the number of cleavage sites and approximate fragment sizes (bp) with the corresponding results from the digestion of BPV-1 and BPV-2 genomes.

RE digest *Hin*dIII +	DoPV		BPV-1		BPV-2	
	No. of sites	Fragment sizes	No. of sites	Fragment sizes	No. of sites	Fragment sizes
*Bam*HI	2	5600, 2400	2	5437, 2508	4	5272, 2031, 607, 27
*Eco*RI	2	3300, 4700	2	3100, 4845	1	7937
*Kpn*I	3	3750, 3550, 700	2	4446, 3499	3	3939, 3633, 365
*Sma*I	2	6200, 1800	2	6011, 1934	1	7937
*Sst*I	1	8000	1	7945	2	5726, 2211
*Xba*I	1	8000	2	7119, 826	2	5565, 2372

L1 ORF

```
                        6970        6980        6990        7000
DoPV                ...TTCTTTG GACTTAGATC AATTTCCCTT GGAAGAAGA TTTTTAGCAC
                    ...||||||| |||||||||| |||||||||| || |||||| ||||||||||
BPV1                ...ttctttg gacttagatc aatttccctt gggagaaga tttttagcac

                    7010        7020        7030        7040        7050
DoPV                AGCAAGGGGC AGGATGTTCA AC.GTGTAGA AACGAAGAAT TAGCC..AAA
                    |||||||||| |||||||||| || |||   | |||||||||| ||||| |||
BPV1                agcaaggggc aggatgttca actgtgagaa aacgaagaat tagccaaaaa

                    7060        7070        7080
DoPV                ACTT..AGTA AGTCCGCAAA AAA...
                    ||||  |||| || | ||||| |||
BPV1                acttccagta agcctgcaaa aaa...
```

E5 ORF

```
                    3910        3920        3930        3940        3950
DoPV          .....TTGGG ACTAGTTGCT GCAATGCAAG TGCTGCTGTT ACTGTTCTTA CTCTTGTTTT
                   ||||| |||||||||| |||||||||  ||||||| || |||||||||| ||||||||||
BPV1          .....ttggg actagttgct gcaatgcaac tgctgctatt actgttctta ctcttgtttt

                    3960        3970        3980        3990        4000
DoPV                TTCTTGTATA CTGGGATCAT TTTGAGTGCT CCTGTACAGG TCT...
                    |||||||||| |||||||||| |||||||||| |||||||||| |||
BPV1                ttcttgtata ctgggatcat tttgagtgct cctgtacagg tct...
```

Figure 17. Nucleotide base sequences of the papillomaviral element DoPV in upper case and BPV-1 in lower case. The DoPV was extracted from sarcoid Do1b and cloned into pIC-20H at the unique *Hin*dIII site. Sequencing was carried out utilising the Sequenase^{r} kit (US Biochmeical Corporation), with Universal M13 primer for the L1 open reading frame (ORF) segment close to the cloning site and custom-synthesised BPV-1 oligonucleotide sequences for the E5 ORF segment. The sequencing reactions were the adapted dideoxy method of Chen and Seeberg (1985) on double-stranded DNA produced by acid-phenol minipreparation. The comparison to BPV-1 illustrated was generated by employing the BESTFIT local homology based on the algorithm of Smith and

6.4. DISCUSSION

The results presented in this chapter have described the detection of full-length papillomaviral genomes, identical or very similar to BPV-1 and 2, in donkey sarcoids. In the present study, the papillomaviral DNA was demonstrated in all 18 sarcoids screened which is a higher rate of detection than both the 12/14 of Trenfield *et al.* (1985) and the 29/33 of Angelos (1990). The papillomaviral DNA was not detected in the non-sarcoid samples derived from a melanoma, granulation tissue and normal skin and these findings suggest that the papillomaviral DNA may be involved in the aetiology of the sarcoid in the donkey.

However, on comparison of the present study with the most recent and largest scale screening of sarcoid tumours (Angelos, 1990), it is apparent that there are several differences between the two studies that are worthy of note, besides the detection rate. First, Angelos (1990) detected papillomaviral DNA in one of two pyogranulomatous tissues, a result which is surprising until consideration is given to the difficulty in confirming a diagnosis in the case of ulcerated sarcoids. There is little doubt that the presentation of a sarcoid biopsy without an epithelial component is one of the more troublesome tasks faced by histopathologists, and, as a consequence, the diagnosis in the case of equine pyogranulomatous tissue may be open to question, especially when papillomaviral DNA is detected. Furthermore, of all the suggested modes of transmission of the sarcoid, the most popular is that the aetiological agent, whatever it may be, gains entry at a site of injury to the integument, and so there would appear to be an argument for a sarcoid developing alongside granulation tissue.

Secondly, Angelos (1990) failed to detect papillomaviral DNA in three confirmed sarcoids, a finding which supported the results of Trenfield *et al.* (1985). Although the suggestion was made that a more sensitive method of detection may have demonstrated the presence of papillomaviral DNA, or that the diagnosis may have been incorrect, a third possibility exists: at some stage in the development (or,

and the subsequent clinical development, as well as histopathological assessment of development or regression would be essential information. In the present study, all sarcoids were positive for papillomaviral DNA and although no estimate was made of tumour age, a wide selection of tumour sizes were studied. If the size and degree of ulceration is at all indicative of lesion maturity, then it may be fair to assume that both reasonably new and long standing tumours were represented. Importantly, in no case were the histopathological changes indicative of tumour rejection, and it would be interesting to examine sections of those sarcoids negative for papillomaviral DNA from Angelos's case series, to ascertain whether or not histological changes associated with tumour rejection were evident.

Thirdly, Angelos (1990) detected partial, as well as full length, papillomaviral genomes in two of the sarcoids studied and the deletions of 25 and 1.9 per cent of the genome were localised to the same region as the 9 per cent deletion reported by Amtmann *et al.* (1980). In the present study all genomes were full length, approximately 8kbp in size, results in agreement with the vast majority of tumours screened in previous studies, suggesting that the partial genome may be the exception to the general finding.

Having established the presence of papillomaviral DNA in the tumours, consideration must be given to the characterisation of the genomes. That the papillomaviral DNA is similar to BPV-1 and 2 is without doubt; probing with BPV-2 under stringent conditions indicated a high degree of homology, results in agreement with previous workers (Lancaster *et al.*, 1977; Lancaster *et al.*, 1979; Amtmann *et al.*, 1980; Lancaster, 1981; Trenfield *et al.*, 1985; Angelos *et al.*, 1991). In the present study, the identities of the genomes detected fell into three classes; one identical to BPV-1; one identical to BPV-2; one similar to both BPV-1 and BPV-2 but identical to neither. Angelos (1990) and Angelos *et al.* (1991), detected BPV-1-like and BPV-2-like DNA in a study involving sarcoids from Switzerland and the USA and Trenfield *et al.* (1985) detected DNA identical to neither BPV-1

and cloned papillomaviruses from equine papillomas, demonstrating different RE fragment patterns in viral DNA isolated from different anatomical sites, as well as varying degrees of homology to BPV-1 and HPV-1. None of the RE fragment patterns observed by O'Banion *et al.*, (1986) resembled the results of the present study. It is unfortunate that the results from previous studies are not exactly comparable as there was no standardised choice of either REs or stringency of hybridisation but, despite the fact that some of the data presented by Angelos (1990) were complicated by partial digestion of some of the DNA samples, it was apparent that the sarcoids screened contained DNA practically identical to BPV-1 and BPV-2, as well as DNA similar to BPV-1 and BPV-2, results broadly similar to the findings outlined in this chapter. However, care is required when interpreting these results as the variation from BPV-1 was slight, a limited number of REs were used in the analysis of the fifteen genomic samples and no multiple-cutters were employed in the study. The novel isolate differed from BPV-1 only in the absence of the *Xba*I site and the presence of an additional *Kpn*I site, the latter being found in BPV-2. Although a complete mapping using multiple double digests was not performed, there was great similarity in the fragment sizes of DoPV and BPV-1 following HindIII/RE digestion and it is a possibility that there may be several wild-type BPV-1 papillomaviruses: Angelos (1990) pointed out the limitations of comparing RE digests alone by the demonstration of an RE digest pattern of DNA from a BPV-2 induced lesion different to that previously published.

The similarity of the DoPV to BPV-1 is further emphasised by the nucleotide base sequence of the cloned element. In both instances there was a high degree of homology with over 95 per cent similarity in the L1, 98 per cent similarity in the E5 and 96.5 per cent similarity combined. However, it should be remembered that only 212 of the 8000bp were involved in the analysis and it may be the case that other parts of the genome are less similar; sequencing of the URR, a region known to show variation between closely related papillomaviruses

E5 ORF is well conserved and there is complete identity of the amino acid sequence in BPV-1 and BPV-2 (Pfister, Krube, Dietrich, Iftner and Fuchs, 1986). However, the comparison of the DoPV sequences with BPV-1 and 2 becomes more significant when the nucleotide base similarity of the two bovine viruses is considered. The results of the limited sequence analysis indicate that there is greater homology between DoPV and BPV-1 than there is between BPV-1 and BPV-2, viruses that are known to be very closely related. The extremely high homology thus supports the similarity of the RE digests and it seems most likely that DoPV is in fact a subtype of BPV-1, in agreement with the conclusions of Trenfield *et al.* (1985) and Angelos *et al.* (1991). The detection of specific E5 sequences confirms the presence in the DoPV of at least one of the ORFs known to have a transforming function in the biology of BPV-1, suggesting that cellular transformation in sarcoids may be associated with this viral oncogene. The presence of other ORFs, such as the E6, requires confirmation by further sequencing or probing with subgenomic fragments, but although similar genomic organistion is suggestive of a uniformity of function, expression and transfection experiments would still be required.

In the case of the L1 fragment, there is also great homology between the DoPV and BPV-1 sequences and although the L1 is relatively well conserved amongst the subgroup A bovine papillomaviruses, minor differences in the L1 may lead to altered structure of the major capsid protein which is coded for by the L1 ORF (Pfister, 1984). It is interesting to speculate that the differences, although minor, may include codon alterations and perhaps frame-shifts, resulting in an immunological epitope that possesses the interspecific antigenic properties of BPV-1 and BPV-2 but has an altered neutralising antibody binding site, one possible explanation for the detection of non-neutralising antibody to papillomaviral antigen in Chapter III.

The great similarity between DoPV and BPV-1 could be confirmed by

An alternative method of confirmation by heteroduplex comparison of BPV-1 and DoPV (Campo and Coggins, 1982) is currently being undertaken with the DoPV recloned into pBR322, and preliminary results have failed to demonstrate any significant areas of reduced homology.

Further work is necessary to investigate the state of papillomaviral DNA in the sarcoid and the consideration of future strategies highlights the deficiencies of this, and previous investigations. In the present study, it was not possible to obtain control healthy skin samples from each of the donkeys with sarcoids, and it may be the case that latent papillomaviral genomes may exist in healthy tissue, awaiting activation by some insult to the integument, as in *Mastomys natalensis* (Amtmann, Volm and Wayss, 1984). In addition, precise mapping in order to localise the RE cleavage sites in the DoPV genome involving, double digests with all possible combinations of the REs used in the present study, a wider range of REs as well as those with multiple cleavage sites, would provide further information about the genomic organisation in relation to the bovine viruses. In this respect, it is unfortunate that the REs used in this study were different to those employed by Angelos (1990) and other workers, thus precluding direct comparison, and an important initiative would be the establishment of standardised protocols in any collaborative multicentre prospective study.

The screening methods outlined at the beginning of this chapter in the detection of papillomaviral genomes in sarcoid tumour cells were time consuming and involved the use of radioactive isotopes, the latter being a hazard to researcher and environment. In addition, substantial biopsies are required from clinical cases in order to provide adequate amounts of DNA for RE analysis and the provision of such biopsies usually requires invasive surgery and was the limiting factor in the failure to obtain control healthy tissue. The following chapter describes the methodology adopted to overcome these drawbacks, allowing rapid non-radioactive screening and RE mapping of DNA extracted from smaller tissue

CHAPTER VII

DETECTION OF PAPILLOMAVIRAL DNA SEQUENCES IN SARCOID TUMOURS USING THE POLYMERASE CHAIN REACTION

CHAPTER VII

DETECTION OF PAPILLOMAVIRAL DNA SEQUENCES IN SARCOID TUMOURS USING THE POLYMERASE CHAIN REACTION

7.1. INTRODUCTION

In order to carry out the analyses described in Chapter VI, tumour biopsy, DNA extraction, restriction endonuclease digestion, Southern blotting and hybridisation were performed. This was a labour intensive process and required the use of radioactive isotopes. The Polymerase Chain Reaction (PCR) is a rapid, sensitive, primer directed method for DNA amplification (Saiki, Gelfand, Stoffel, Scharf, Higuchi, Horn, Mullis and Erlich,1987), involving the use of a thermostable DNA polymerase and the repeated dissociation and annealing of selected oligonucleotide primers to a DNA template, in the presence of substrate. Using either specific or consensus primers, this technique has been used extensively in human papillomaviral research as a means of detecting papillomaviral DNA from very small tumour biopsies (Levi, Delcelo, Alberti, Torloni and Villa, 1989; Melchers, van der Brule, Walboomers, de Bruin, Burger, Herbrink, Meijer, Lindeman and Quint, 1989). The quantities of DNA generated are typically sufficient for restriction endonuclease analysis, the results of which can be visualised on an ethidium stained agarose gel, without the need for Southern blotting and hybridisation. The papillomavirus genome contains a number of interspecies conserved sequences (Law, Lancaster and Howley, 1979; Danos *et al.*, 1984; Baker, 1987) and the use of such sequences in primer design for use in a

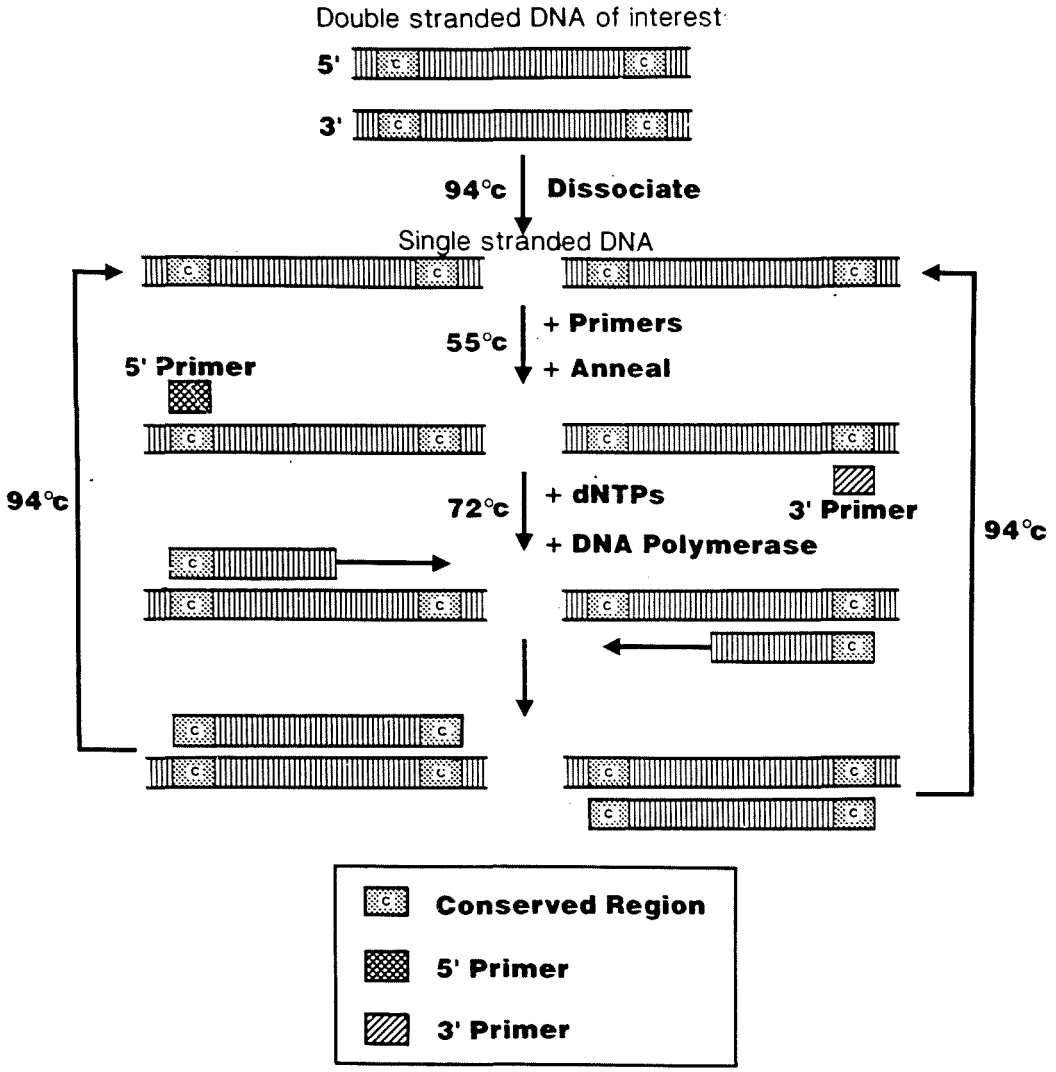


Figure 18. Schematic representaion of the polymerase chain reaction showing the use of consensus primers in the amplification of a fragment of DNA lying between two conserved regions of the

As outlined in the previous chapter, papillomaviral DNA has been detected in DNA derived from sarcoid tumour cells (Lancaster *et al.,* 1977; Lancaster *et al.,*1979; Amtmann *et al.,* 1980, Lancaster, 1981; Trenfield *et al.,* 1985, Angelos *et al.,* 1991) but not in every case. Trenfield *et al.* (1985) demonstrated the presence of episomal papillomaviral DNA that co-migrated with bovine papillomavirus types 1 and 2 (BPV-1 and 2) in 12 out of 14 cases and Angelos *et al.,* (1991) in 29 of 33 sarcoids. The presence of the papillomaviral sequences did not appear to be related to either the clinical type of the lesion or the presence or absence of the epidermis. Restriction endonuclease analysis of the DNA extracted from the sarcoid has been shown to differ from that of BPV-1 and 2 but only at subtype level (Trenfield *et al.* 1985), findings confirmed by Angelos *et al.* (1991).

The aims of this part of the project were to use consensus primers and the polymerase chain reaction to test for the presence of papillomaviral sequences in the DNA extracted from sarcoid tumours; to confirm the identity of the amplified products by Southern blotting and hybridisation; to analyse the PCR products by restriction endonuclease digestion; to confirm the results obtained by Southern hybridisation of genomic digests presented in the previous chapter.

7.2. MATERIALS AND METHODS.

7.2.1. Source of tumours

Sarcoid tumour biopsies were obtained from 18 sarcoid-affected donkeys at the Donkey Sanctuary, Sidmouth, Devon. Biopsies were snap frozen in liquid nitrogen and stored at -70°C until required for DNA extraction.

7.2.2. Preparation of genomic DNA

Extraction of genomic DNA was performed essentially as described in Section 5.3.3., with minor modifications. Briefly, tumours were macerated with a scalpel

SDS to a final concentration of 0.5 per cent w/v. The mixture was then incubated at 37°C overnight. The aqueous phase was then extracted three times with SS-phenol/chloroform and once with diethyl ether. The DNA was precipitated by the addition of two volumes of absolute ethanol and removed by spooling out with a sealed pasteur pipette. Following a brief wash in 70 per cent ethanol, the DNA was dried briefly under vacuum and resuspended in TE (pH 8.0).

7.2.3. Design of consensus oligonucleotide primers

Oligonucleotides 15 bases in length were selected from the interspecies conserved regions in the E1, L2 and L1 open reading frames (ORFs) of BPV-l, BPV-2, Human PV-6 and the European Elk PV (Table 27.). The oligonucleotides were synthesised on an Applied Biosystems 380A DNA Synthesiser using the beta-cyanoethyl phosphoramidite method and purified as described in Section 5.3.10..

Table 27. Nucleotide base sequence of the six oligonucleotide primers and open reading frame (ORF) in which the sequences were identified. The strand to which the primers were compilmentary is also shown. The primers were used in pairs, i.e., 1 and 2; 3 and 4; 5 and 6.

Primer No.	Sequence	ORF
1	TTC GGA TCC ATG GTG	E1 (5')
2	CCT GAA TTC CAA GTC	L2 (3')
3	TTC GAA TTC CTA TGA	L2 (5')
4	CAG AAG CTT GCA GGA	L1 (3')
5	CTG AAG CTT TAG GTA	L2 (5')
6	AAG CTT AGG ATC ATA	E1 (3')

7.2.4. The Polymerase Chain Reaction

The PCR was performed as described by Saiki *et al.* (1987) using the GeneAmp PCR Reagent Kit (Perkin Elmer Cetus). Ten ul of 100ng/ul template DNA from the sarcoid tumours was added to a 40ul reaction mixture containing, 200uM of dATP, dCTP, dGTP, dTTP, 50pmol of both primers from the pairs described above, 1 unit of Amplitaq DNA Polymerase and 5ul of reaction buffer. The reaction buffer contained 100mM Tris HCl, 500mM potassium chloride, 0.01 per cent (w/v) gelatin and either 5, 10, 15, 20, 25 or 30mM magnesium chloride. Ten ul aliquots of ultrapure water, TE (pH 8.0), ovine lymphocyte DNA (100ng/ul) and lymphocyte DNA from a clinically healthy donkey were used as negative controls. The positive control was 10ul of BPV-l virion DNA (5ng/ul). The solution was overlaid with two drops of mineral oil to prevent evaporation. Forty cycles of amplification were performed using a Perkin Elmer Cetus thermal cycler. Each cycle consisted of 1 minute at 94°C to denature the DNA, 1 minute at 55°C to anneal the primers to the template and 3.5 minutes at 72°C for primer extension. After the last cycle, a further incubation for 7 minutes at 72°C was performed to allow extension of any partially completed product.

7.2.5. Analysis of PCR products

On completion of the amplification, 10ul of the reaction mixture was electrophoresed through a 1.5 per cent LMP agarose gel. The DNA was visualised by staining with ethidium bromide and exposure to ultraviolet light (312nm). Amplified products were recovered from the gel as described in Section 5.3.5., phenol/chloroform extracted, ethanol precipitated and resusupended in TE (pH 8.0). One ug of each fragment was then incubated with 3 units of *Hin*dIII, *Bam*HI and *Kpn*I restriction endonucleases in the appropriate reaction buffer for 1 hour at 37°C. Agarose gel electrophoresis was performed as described above. The agarose

blotting in 0.4M sodium hydroxide. The blots were air dried. Prehybridisation and hybridisation were carried out at 42°C for 8 and 16 hours respectively in a solution containing 5x SSC, 5x Denhardt, 0.5 per cent SDS, 75mM EDTA and 0.1mg/ml denatured salmon sperm DNA. The probe was full length molecularly cloned BPV-2 genome labelled with [alpha-^{32}P] dCTP, using the Multiprime DNA labelling system (Amersham International plc). The blots were washed twice for 20 minutes at 42°C in 2x SSC, 0.1 per cent SDS and twice for 30 minutes at 65°C in 0.5x SSC, 0.1 per cent SDS. The blots were exposed for 4 hours to Kodak XAR Omat film at -70°C.

7.3. RESULTS

Amplified products were generated in all 18 cases using the three primer sets. The fragments generated were approximately 2450 (E1-L2), 2650 (L2-L1) and 2900 (L1-E1) base pairs in length (Figure 19). All fragments hybridised with full length molecularly cloned BPV-2 under stringent conditions (Figures 19 and 20). The negative controls yielded no detectable products. Restriction endonuclease analysis revealed the absence of a *Kpn*I restriction site in the L1-E1 fragment in three of the samples (Figure 20). An additional *Bam*HI site in the L2-L1 fragment was present in four of the 18 samples, one of which also lacked the *Kpn*I site in the L1-E1 fragment. In all cases the DNA fragments could be seen on the ethidium stained agarose gel (Figure 21) and it was found that the assay was of optimal sensitivity with fewest artefactual bands when the concentration of Mg^{2+} was 1.5mM.

7.4. DISCUSSION

In this chapter the polymerase chain reaction has been used to detect papillomaviral DNA in cryo-preserved biopsies from equine sarcoids. The PCR is

contamination of genomic samples with cloned papillomaviral plasmids or PCR products. In order to eliminate this possibility, extraction of genomic DNA, preparation of the PCR reaction mixtures, amplification and analysis of the products were carried out in different laboratories. In addition, all PCR products were aliquoted prior to the study. Where possible, screw-top microcentrifuge tubes were used in order to avoid the aerosolisation of PCR products. In the present study, papillomaviral DNA was detected in all cases of histologically confirmed sarcoids. The amplified products were visible on agarose gels stained with ethidium bromide and viewed under ultraviolet light without the need for hybridisation, although Southern blotting and hybridisation with radiolabelled BPV-2 was used to confirm the identity of the products. This is an improved rate of detection compared with the findings of Trenfield *et al.* (1985), Angelos (1990) and Angelos *et al.* (1991), and is in agreement with the rate of detection demonstrated by Teifke and Weiss (1991). The data agree with the findings of the previous chapter that papillomaviruses are implicated in the aetiology of sarcoids in the donkey and although the detection rate was 100 per cent by Southern blot, much smaller amounts of tumour DNA were required for a positive result using PCR, typically 1ug in the case of PCR versus 10-15ug for Southern blot an hybridisation. The use of consensus primers from conserved regions of the papillomaviral genome thus provides a rapid, sensitive method of screening a large number of tumours for the presence of papillomaviral DNA.

It has been shown that in the region between L1 and E1 a *Kpn*I restriction site was absent in four of the 18 samples assayed thus localising the additional *Kpn*I site in DoPV described in the previous chapter to the region between the *Hin*dII site and the origin, a site approximately in the same region as in BPV-2. The presence of an additional *Bam*HI site provides more evidence that several subtypes of papillomavirus may be involved in the aetiology of the disease, adding

to the results of Teifke and Weiss (1991). In the latter study, primers from the E5 ORF of BPV-1/2 were used to amplify a region of the viral genome that in general exhibits a high degree of variability between papillomaviral types but is relatively well conserved amongst the fibropapillomas. Restriction endonuclease digests of the products using *Bst*I, showed all amplification products to be indistinguishable from BPV-1, a result which supports the high nucleotide base sequence homology described in the preceding chapter. The results of the present study have demonstrated minor differences from the restriction pattern of BPV-1, but, as a limited number of restriction enzyme cleavage sites were utilised, more extensive mapping using a larger number of restriction enzymes and multicutting enzymes may distinguish the DNA into further subtypes. In addition, type specific primers may be used to screen for different subtypes (Nuovo, Darfler, Impraim and Bromley, 1991) and PCR products may now be sequenced directly (Gyllensten, 1989), thus removing the need for many of the procedures described in the previous chapter.

The involvement of papillomaviral DNA in sarcoids using PCR may be investigated further by relating the subtype of viral DNA to tumour type, anatomical site and geographical location. PCR has also been applied by several workers to the detection of papillomaviral DNA in formalin fixed paraffin-embedded tissue sections (Claas, Melchers, van der Linden, Lindeman and Quint, 1989; Shibata, Gupta, Shah, Arnheim, and Martin, 1988). This will allow extensive archival studies to be carried out, work currently being undertaken as an extension of the current project. In the present study, one of the major limitations was that the majority of samples assayed were from clinically diseased animals and undoubtedly, other tissues, healthy and diseased, should also be screened for the presence of papillomaviral DNA. This can now be carried out without the need for large skin biopsies from normal donkeys due to the sensitivity

at The Donkey Sanctuary.

Although the PCR is undoubtedly a powerful investigative tool, interpretation of data resulting from such studies cannot be divorced from either clinical or epidemiological studies. Wikstrom, Lidbrink, Johansson and von Krogh (1991) employed PCR in screening males attending a sexually-transmitted diseases clinic, for the presence of HPV 6 or 16. Using both dot blot and agarose gel methods of analysis these authors demonstrated a detection rate of 76 and 43 per cent for HPV-6 and 16, respectively, in men with no previous history or evidence of condylomata. Furthermore, a study of clinically normal women undertaken by Tidy, Vousden and Farrell (1989) demonstrated HPV 16 DNA in over 80 per cent of individual. These studies suggest that the frequency of occult papillomaviral infections, as demonstrated by PCR, is extremely high, but in the case of HPV 16, the over representation of the viral type in comparison to the prevalence in clinical lesions, suggests that extreme caution is required before making any definitive conclusion, far less clinical decision or diagnosis, based on PCR data alone.

The drawbacks of PCR diagnosis are well documented (Stanley, 1990) but in HPV research the technique is rapidly becoming the preferred method of identification (Guerrero and Shah, 1991). However, with the introduction of standardised reagents, protocols designed to avoid contamination and the development of quality controls (Guerrero and Shah, 1991) PCR is a valuable component in the diagnostic armamentarium of a mutidisciplinary approach to disease investigation (Wright and Winford-Thomas, 1990).

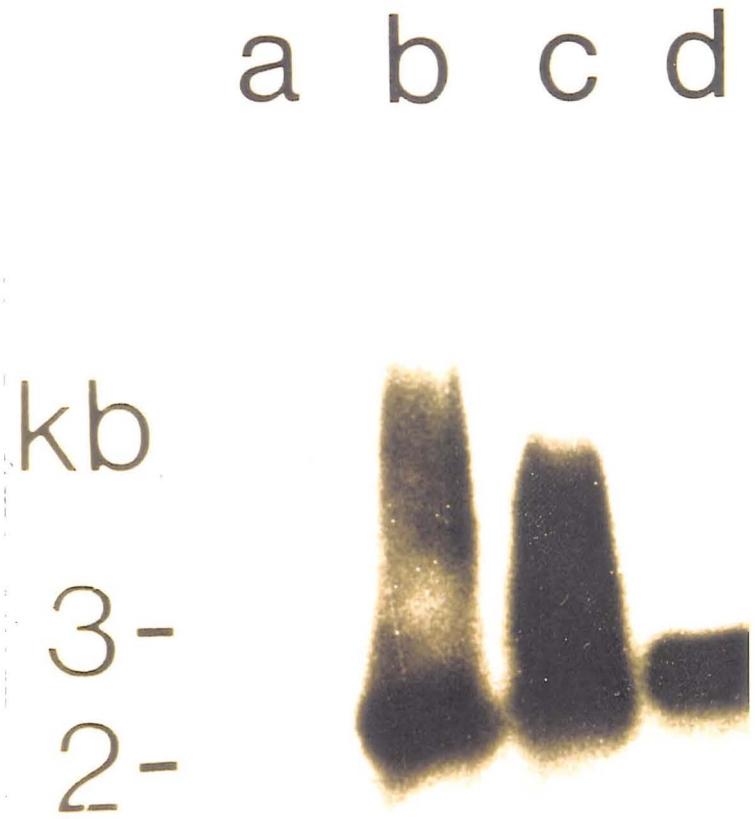


Figure 19. Autoradiograph of a Southern blot after hybridisation showing the amplified products of the three fragments of the papillomaviral genome as detected in all tumour samples. a, Normal equine skin DNA template with primers for El-L2; b,c and d, sarcoid DNA template with primers for E1-L2, L2-L1 and L1-E1, respectively. Hybridisation was to molecularly cloned BPV-2 DNA.

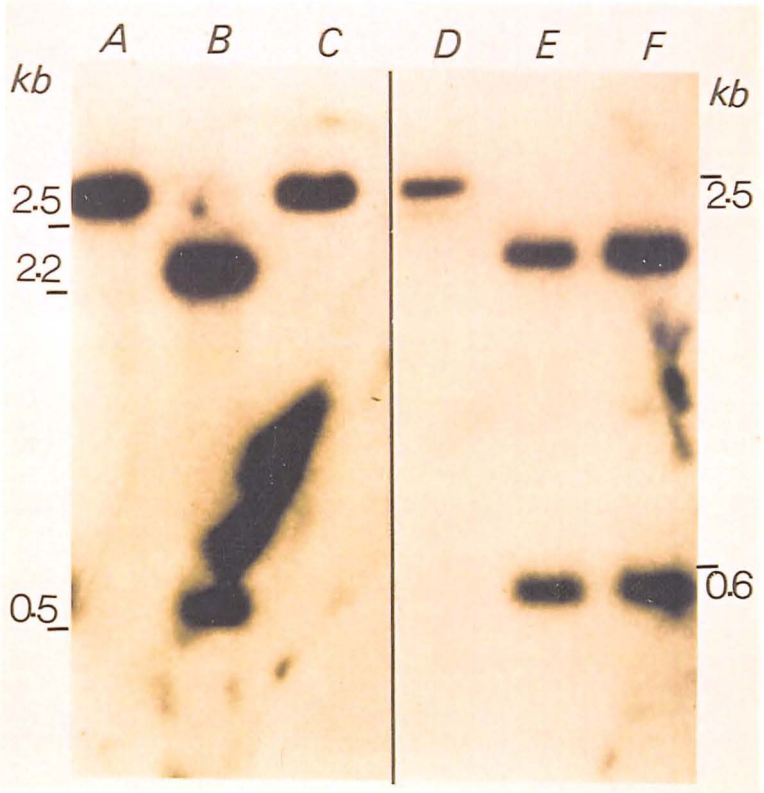


Figure 20. Autoradiograph of a Southern blot showing *Kpn*I restriction endonuclease digestion of two subtypes of papillomaviral DNA amplified by PCR. A, undigested L1-E1 fragment; B and C, *Kpn*I digested L1-E1 fragment showing absence of *Kpn*I site in sample C. D, undigested E1-L2 fragment; E and F, *Kpn*I digested E1-L2 fragment showing presence of *Kpn*I site in both fragments.B and E were products from the same sarcoid DNA template, C and F were both products from a different sarcoid DNA template. Hybridisation was to molecularly cloned BPV-2 DNA.

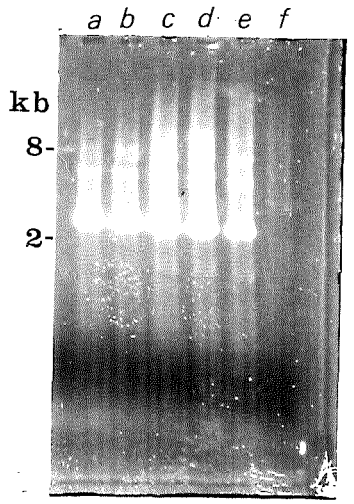


Figure 21. PCR products after electrophoresis through a 0.8 per cent agarose gel showing effect of Mg^{2+} concentration. Each lane was loaded with 10ul of PCR products following a 40 cycle amplification of 1ug DNA extracted from a sarcoid, employing oligonucleotide primers from the E1 and L2 open reading frames of BPV-1.. The magnesium ion concentrations used were (a-e) 1.0, 1.5, 2.0, 2.5 and 3.0mM with 1.5mM Mg^{2+} judged to provide clearest bands with fewest artefacts. The 2.5kbp product was visible without the need for Southern blot, hybridisation and autoradiography. Lane f contained the negative control assyed at 1.5mM Mg^{2+}. The gel was stained with ethidium bromide solution and photographed when transilluminated with UV light (312nm).

CHAPTER VIII

GENERAL DISCUSSION AND SUMMARY

CHAPTER VIII

GENERAL DISCUSSION AND SUMMARY

The aim of this research project was to investigate aspects of the equine sarcoid of the donkey, with the long term objective of assessing the feasibility of adopting a vaccination programme against the disease at The Donkey Sanctuary. The first stage in the investigation was into the epidemiology of the disease, identifying animals with sarcoids and those most at risk of developing the disease. The results of Chapter II are a major advance in this respect and have provided the basis for management changes at The Sanctuary. Based on the investigation into the epidemiology of the disease at The Sanctuary, a disease model has been developed and, *en route* to this model it has been demonstrated that several variables affect the likelihood of individual donkeys having sarcoids. Sex, age at entry and exposure have all been shown to have individual effects as well as interaction of sex and exposure effects and this has highlighted the young castrated males as being the group of animals most likely to have sarcoids.

Any procedures requiring surgical intervention and general anaesthesia carry a risk of death or sequelae detrimental to the health of the animal and, in order to minimize the suffering of the donkey, removal of tumours should be performed as soon as possible after diagnosis, reducing stress and physical trauma. Furthermore, as all the Case animals are treated by a variety of modalities and in most instances require a general anaesthetic, the disease is of economic significance to The Sanctuary's financial budget. In general terms, the duration of the operation and the financial cost to the charity is related to the size and number of tumours. Thus, minimising trauma and expense may best be achieved by the

have been identified and changes in management implemented, ensuring regular inspection of the young male donkeys by the paraclinical staff at The Sanctuary. The high risk groups at The Sanctuary are now being examined monthly whilst those less at risk are examined less frequently, identifying those animals with sarcoids at an earlier stage and allowing the therapeutic measures currently being employed by the clinicians at The Donkey Sanctuary to be implemented sooner in the disease process. As surgical procedures are now carried out sooner, earlier lesions are being submitted for histological examination which, whilst improving the welfare of the donkeys, also provides valuable material for further investigation, not available previously. This will lead to a fuller understanding of the development of the tumours and the role of aetiological agents in the pathogenesis of the disease.

Other specific managemental changes have arisen from the observation that a possibility exists, suggested by the sex, age and exposure of the animals most affected by sarcoids, that a potential pathogen may be disseminated at, or around, the time of castration. It has been recommended that strict attention should be paid to complete sterilisation of surgical instruments between operations, including cryoprobes and cautery blades, and that insecticidal sprays and insect repellents should be used to minimise the possibility of fly-borne transmission of an aetiological agent, either on the same animal or to a close in-contact which may share a box immediately post-operatively. It may also be advisable to keep all scrotal wounds covered with surgical dressings until healing has occurred, but this may prove to be impractical.

The epidemiological study described has underlined the immense potential for developing the database management of animal disease in the population of donkeys at The Donkey Sanctuary. The construction of disease-risk models for other diseases, using carefully structured databases and the interrogative software tools employed during the current project, will lead to the identification of major

Having identified a diseased population and animals at risk from the disease, the success of a vaccine development requires the identification of an aetiological agent. The demonstration of papillomaviral DNA in the sarcoid material collected from the animals at The Sanctuary is strongly suggestive of the involvement of this known pathogen in the aetiopathogenesis of the disease, results in agreement with the conclusions of other workers. The findings of Jarrett *et al.* (1990a; 1990b; 1991) in vaccination studies against BPV-induced neoplasms in bovidae, indicate that there is a place for both therapeutic and prophylactic vaccination strategies against papillomaviral infection. However, assuming the involvement of a papillomavirus in the donkey sarcoid, the differences between the disease process in the two species must be highlighted. First, the apparent absence of a productive papillomaviral infection in the donkey is in contrast to the production of infectious viral particles in the bovine cutaneous lesions. Secondly, infection of cattle by BPV-1 and 2 results in an humoral immune response with the production of neutralising antibody. In the donkey this does not appear to be the case, although the results of the Western blot assays in Chapter II demonstrate an antibody response to a 64 and 55kDa antigen similar in size to the L2 and L1 viral proteins of BPV-1 and 2. The absence of neutralising antibody is in agreement with the findings of Ragland and Spencer (1968) but is in contrast to the presence of neutralising antibody in horses inoculated intradermally and subcutaneously with BPV (Segre *et al.,* 1955; Olson and Cook, 1951). Thirdly, in cattle the tumours are usually rejected in 4-12 months, whereas in the donkey the lesions persist. There are no clinical records at The Sanctuary that suggest any of the donkeys have rejected the tumours.

The apparent lack of viral production must be considered in more detail. Viral extractions performed on the donkey tumours were usually carried out on mature lesions larger than $1cm^3$ in size. A very early horse sarcoid did yield papillomavirus-like particles and it may be the case that virus is produced in the

production has ceased. In order to investigate whether or not viral production occurs at any stage in the development of the sarcoid, more vigilant attention must be paid to the animals in the high risk categories, identified in Chapter II, in the hope of observing, biopsying and assaying earlier lesions for the presence of virions. It is also important that any attempt at viral extraction should be from a field case, as it is known that the biology and pathology of the disease produced by experimental infection of equine skin with BPV-1 and 2 differs from the natural disease.

The need to isolate an infectious particle and fulfil Koch's postulates remain and Ragland *et al.* (1970b) stated that the understanding of the equine sarcoid is unlikely to be advanced until suspected virus(es) have been isolated and characterised. The cloning and partial characterisation of a papillomaviral genome described in Chapter VI and the isolation of a viral particle in Chapter IV (albeit from a horse sarcoid) provide the basis for more detailed molecular biological and viral investigations. In the face of the accumulating evidence demonstrating papillomaviral DNA in sarcoid tumours throughout the world, (Lancaster *et al.*,1979; Amtmann *et al.*, 1980; Trenfield *et al.*, 1985; Angelos *et al.*, 1991) it seems increasingly unlikely that the virus does *not* play a role in the aetiopathogenesis of the tumour. Futhermore, the evidence presented in Chapters VI and VII confirms previous reports that although the viral DNA isolated is very similar to BPV-1 and 2, subtypes of the viruses may be involved in the disease process.

The development of an assay for papillomaviral DNA using PCR technology (Chapter VII) allows equine tissue samples to be screened rapidly and without the need for Southern blotting or the use of radioactive isotopes. This technology is currently being applied to other equine tissues such as PBLs and formalin-fixed paraffin-embedded tissue sections and information from these studies may provide insight into latency of infection involving PBLs as suggested by

The development of a PCR assay is another major step towards improved welfare of affected donkeys as it is now possible to take small amounts of tissue and screen for papillomaviral DNA. During the course of the present study, histopathological confirmation of sarcoid tissue was carried out by examining formalin-fixed paraffin embedded tumour sections stained with haematoxylin and eosin. It was noticed that in distinction to bovine cutaneous lesions the hair follicle was very frequently involved in the tumour of the donkey, with marked proliferation of the epidermis around the hair follicle. Thus, plucking a few hairs from the tumours may provide enough material for PCR analysis and it may now be possible to provide control tissue for screening without the need for surgical biopsy of healthy skin, a procedure precluded by the constraints of the current project. Another interesting application of the exquisite sensitivity of PCR would be to assay DNA from insect vectors trapped at The Sanctuary in order to ascertain whether papillomaviral DNA was present and thus go some way to confirming fly-borne transmission as one route of inoculation of a putative aetiological agent.

Ultimately, any future research must be based on a multidisciplinary approach to the disease involving concurrent investigation into both aetiological and host factors. The early transmission and serological studies were carried out before the existence of different types and sub-types of BPV was known. However, it is most likely that the viruses involved were either BPV-1 or 2 and although a case could be made for the repetition of the experiments with known amounts of typed virus, it is unlikely that the results would be radically different from those published. In contrast, the need to consider the host in the response to experimental inoculation of either homogenised tumour or BPV should be given priority. Genetic predispostion to sarcoids, evidenced by the association of specific ELAs (MHC types) to risk of the tumour in the horse, may well explain the inconsistent results of the various workers who have tried to reproduce the disease

Unfortunately, the small amount of familial information available concerning the animals involved in this study was too sparse and unreliable to make any definitive statements with regard to the genetic effect of the disease in donkeys. In view of this, a logical development of the research programme at The Donkey Sanctuary would be to look for disease-risk association with the donkey MHC. This programme of work has recently started using commercially available murine and human class II DNA probes on digests of genomic DNA from the Case population and on a population of paired controls, matching each case for sex and age.

Ragland and Spencer (1969) stated that transmission attempts with nucleic acid rather than tumour extract may provide further information regarding the aetiology of the disease and this may now be carried out comparing whole virus, viral DNA or molecularly cloned viral DNA. Angelos (1990) proposed three models upon which the significance of papillomaviral DNA and host factors could be assessed. In the first, only the fibroblasts of genetically susceptible animals become infected by an ubiquitous papillomavirus; in the second, all fibroblasts of donkeys are infected if they encounter a papillomavirus, but only those fibroblasts of genetically susceptible animals are permissive for papillomaviral replication; in the third model, all fibroblasts of donkeys are infected if the cells encounter a papillomavirus and replication occurs but in resistant animals the transcription of papillomaviral genes is inhibited. All three of these models could be tested *in vitro* on horse-derived fibroblasts for which the host genotype has been characterised, unlike the situation in the donkey.

There are some aspects of the results that require further consideration. If the donkeys are infected with a papillomavirus similar to BPV-1 and 2, why is the antibody produced unable to neutralise the virus in the same manner that horses inoculated with BPV develop neutralising antibody? As suggested in Chapter VI there may be slight differences in the virus that is infecting the donkeys, perhaps traceable to nucleotide base sequences in that part of the viral genome which

likelihood that major immunological differences exist between the two viruses seems remote but cannot, at this stage, be ruled out.

Another possibility lies in the amount of virus required to cause clinical disease. If the infecting dose is very small, it may be insufficient to trigger a response by the immune system, thus allowing the viral DNA to effect transformation and, as there does not appear to be production of viral particles in the donkey tumours examined, no further stimulation of the immune system by the L1. If this explanation is adopted then it must be assumed that the antibody detected in Chapter III is not related to the viral infection causing the disease, but may be due to exposure to another papillomaviral antigen.

Given the similarity of the isolated genomes to BPV-1 and 2 it is surprising that it has not been possible to reproduce the disease experimentally. This may be due to the other factors such as the size of inoculum; in experimental infection it is likely to be many fold larger than a naturally-occurring infection and may consequently interact with the host immune system in a different manner. This would certainly be one explanation for the development of neutralising antibody to BPV-1 in some experimental studies but not in others, nor in animals with sarcoids. However, regardless of whether the development of an humoral immune response is nonexistent, non-neutralising or unpredictable, the economics and ethical considerations of vaccinating prophylactically may be of more importance in the assessment of the feasibility of such an approach: although the sarcoid is among the most common of skin tumours affecting equidae, the disease does not appear to be a risk to all animals equally nor life-threatening to the majority of the general population, and, in view of this, a therapeutic vaccine would seem to be more appropriate to the clinical situation. The work reported by Jarrett *et al.*, (1991) Meneguzzi *et al.*, (1991) and Campo (1991) would indicate that the L2, and transforming early proteins offer possibilities for vaccines that stimulate cell mediated immunity (CMI) resulting in tumour regression and rejection. Campo

During the current project, an attempt was made to demonstrate viral structural proteins in sarcoid tissue sections using anti-BPV-2 peroxidase-antiperoxidase immunocytochemistry, without success, a result that may be expected in view of the failure to isolate virions in the vast majority of tumours. However, as all the tumours in this study were found to contain DNA similar to BPV-1 or 2, one future strategy may be to screen the tissue sections using monoclonal antibodies to BPV-1/2 L1, L2, E5 and E6 proteins. If the virus undergoes only the early part of the replicative cycle, sufficient to produce only the early transforming proteins (E5 and E6), vaccination against these proteins may prevent or halt transformation and stimulate a CMI response leading to tumour rejection.

Other avenues remain to be investigated; the expression of papillomaviral antigens on the cell membrane of transformed fibroblasts *in vitro* and *in vivo*; more complete mapping of the cloned genome; the use of the PCR to screen for specific subtypes; relation of subtype to clinical type, anatomical site or geographical location; localisation of the viral DNA within the tumour either by dissection and extraction of the nucleic acid or by *in-situ* probing of tumour sections with BPV DNA probes; extraction and characterisation of mRNA may provide an insight into the viral biology within the tumour. In conclusion, despite all the suggested scope for future research, there is still a lack of basic knowledge about sarcoids that stems from the inability to reproduce the disease and study it from infection through lesion development to (rarely) rejection. Although clinically-based case-control trials, employing the molecularly cloned proteins developed for the successful vaccination studies in cattle, may provide one area for development, there is still a case for transmission and infection studies both *in vitro* and *in vivo.* Although this work can be performed in the horse, where MHC type has been defined, the same is not true of the donkey and the characterisation of the donkey MHC is an essential development in the research programme. This investigation

animal welfare by identifying donkeys susceptible to the sarcoid.

APPENDIX I

CONTROL POPULATION DATA

Key: **Sex 1; male (gelding)**

Sex 2; female (jenny)

***; Dead donkey (See Appendix IV)**

Number	Sex	Date of Birth	Date of Entry
1	2*	01/01/63	02/28/73
2	2	01/01/65	02/28/73
3	2*	01/01/69	03/13/73
4	2	01/01/64	03/13/73
6	2*	01/01/63	03/13/73
7	2	01/01/69	03/13/73
8	2*	01/01/60	03/13/73
9	2*	01/01/53	05/05/73
10	2*	01/01/53	05/07/73
11	2*	01/01/69	03/13/73
12	2	01/01/68	05/09/73
13	1	01/01/72	05/07/73
14	2	01/01/55	05/08/73
15	2*	01/01/69	05/07/73
16	1*	01/01/66	06/06/73
18	1*	01/01/68	06/19/73
19	2	01/01/63	06/19/73
22	1*	05/01/72	06/19/73
25	1*	01/01/63	06/19/73
27	2	01/01/46	06/19/73
28	2*	01/01/70	06/19/73
30	2	01/01/72	06/19/73
33	1	01/01/68	06/19/73
34	1*	01/01/70	06/19/73
35	2*	01/01/70	08/22/73
36	2	01/01/69	08/22/73
38	2	01/01/63	08/22/73
39	2*	01/01/63	08/22/73
41	2	01/01/70	08/22/73
42	2*	01/01/47	10/11/73
43	2*	01/01/40	09/11/73
44	2*	01/01/59	08/11/73
45	2	01/01/65	09/11/73
47	2*	01/01/38	01/01/68
48	1*	01/01/71	12/12/73
50	2*	01/01/73	12/11/74
52	1*	01/01/58	12/12/73
53	2*	01/01/63	12/12/73
55	1	09/01/73	09/30/73
58	1*	02/01/74	02/24/74
59	2	03/01/74	03/27/74
60	2*	01/01/62	03/27/74
61	2	01/01/74	03/27/74
63	1*	01/01/67	03/27/74
64	2*	01/01/60	03/27/74
65	2*	01/01/59	03/27/74
66	1*	04/01/73	04/21/73
67	2*	01/01/66	04/04/74
69	1*	01/01/53	04/10/74
73	2*	01/01/69	04/10/74
74	2*	01/01/71	04/10/74
78	1*	01/01/65	05/09/74

Number	Sex	Date of Birth	Date of Entry
87	2	01/01/71	05/01/74
88	2	01/01/71	05/29/79
89	1*	01/01/70	05/29/74
90	2*	01/01/60	05/29/74
92	1	05/01/74	05/14/74
93	1*	01/01/70	07/24/74
94	2*	01/01/65	07/24/74
95	2*	01/01/54	07/24/74
96	2*	01/01/73	07/24/74
97	2*	01/01/70	07/24/74
99	2*	01/01/67	07/24/74
100	1*	01/01/69	04/27/74
101	2*	01/01/44	07/24/74
102	2*	01/01/54	07/24/74
103	1*	01/01/60	07/24/74
105	2*	01/01/58	07/24/74
106	2*	01/01/73	07/24/74
107	2*	01/01/70	07/24/74
108	2	01/01/70	07/24/74
109	2	01/01/70	07/24/74
110	1*	01/01/60	07/24/74
112	1*	01/01/54	07/27/74
113	1*	01/01/70	07/24/74
114	1*	01/01/70	07/24/74
115	1	01/01/70	07/24/74
116	2	01/01/66	07/24/74
117	1	01/01/68	07/24/74
120	1*	01/01/68	07/24/74
121	1*	01/01/40	07/24/74
122	1*	01/01/66	07/24/74
124	1	01/01/68	07/24/74
129	1	01/01/70	07/24/74
134	2*	01/01/65	07/24/74
135	1	01/01/59	07/24/74
136	1*	01/01/57	07/27/74
138	1*	01/01/60	07/24/74
139	1	01/01/44	07/24/74
141	1	01/01/68	07/24/74
143	1	01/01/62	07/24/74
144	2*	01/01/60	07/24/74
145	1*	01/01/69	07/24/74
148	1*	01/01/64	09/04/74
150	1	01/01/70	09/10/74
151	2	01/01/70	09/10/74
152	2*	01/01/69	09/10/74
153	1*	01/01/54	09/10/74
154	1	01/01/69	09/10/74
155	1*	01/01/68	09/10/74
156	2*	01/01/49	09/10/74
157	1*	01/01/40	09/10/74
159	2	01/01/73	09/10/74
161	1*	01/01/69	09/10/74

Number	Sex	Date of Birth	Date of Entry
168	1	01/01/68	09/10/74
169	1*	01/01/60	09/11/74
170	1	01/01/72	09/11/74
171	1*	01/01/67	09/11/74
172	1	01/01/68	09/11/74
173	1	01/01/69	09/11/74
174	1*	01/01/68	09/11/74
175	1	01/01/70	09/11/74
176	1	01/01/70	09/11/74
177	1*	01/01/60	09/11/74
178	1*	01/01/67	09/11/74
179	1	01/01/54	09/11/74
181	2*	01/01/71	10/19/74
182	1	01/01/70	11/05/74
183	2*	01/01/25	11/05/74
186	1*	11/01/74	11/01/74
187	2*	01/01/64	12/03/74
188	2*	01/01/62	12/02/74
189	1*	01/01/70	12/12/74
190	2*	01/01/60	12/12/74
192	1	01/01/72	01/07/75
193	1*	01/01/54	12/01/74
194	1*	01/01/55	01/30/75
195	1*	01/01/64	01/30/75
196	1*	01/01/64	04/24/74
198	1*	01/01/67	01/01/74
201	2	01/01/57	06/20/77
202	1*	01/01/70	01/01/74
203	1	01/01/60	07/29/85
204	1*	01/01/68	11/01/74
205	1*	01/01/72	07/29/85
206	1	01/01/63	03/12/80
207	1*	01/01/71	02/01/75
208	1*	01/01/70	02/01/75
209	2*	01/01/55	02/01/75
210	2*	01/01/70	02/01/75
211	1*	01/01/73	02/03/75
212	1*	01/01/70	02/05/75
214	2	01/01/67	02/04/75
216	2	01/01/71	02/04/75
217	2*	01/01/74	02/04/75
220	2	01/01/72	02/04/75
221	2	01/01/66	03/04/75
223	2*	01/01/72	08/01/77
226	1*	01/01/70	03/01/75
228	2*	05/01/63	03/05/75
229	1*	01/01/50	03/19/75
230	2*	01/01/68	03/25/75
231	2	01/01/74	03/25/75
232	2*	01/01/63	03/25/75
234	1*	01/01/74	03/25/75
235	2	01/01/66	04/02/75

Number	Sex	Date of Birth	Date of Entry
241	1*	08/01/73	08/15/73
242	2*	01/01/63	01/01/75
244	2	01/01/68	05/01/75
245	2	11/01/71	05/01/75
246	2*	01/01/69	06/20/74
249	2	01/01/67	01/01/75
250	2*	01/01/75	01/01/75
251	1*	01/01/65	12/06/79
252	2	07/01/70	07/07/70
255	1*	01/01/70	04/01/75
257	2	01/01/73	05/01/75
258	2	01/01/74	05/02/75
259	2	01/01/68	05/01/75
260	2*	01/01/70	05/01/75
261	1	01/01/71	05/01/75
262	2*	01/01/68	05/01/75
264	2	01/01/71	05/01/75
265	2	01/01/73	05/01/75
266	2	01/01/68	05/03/75
268	1	04/01/75	04/29/75
269	2	05/01/75	05/10/75
271	2	05/01/75	05/02/75
272	2*	01/01/70	04/20/75
276	2*	01/01/45	06/10/75
277	2*	01/01/55	06/09/75
279	2*	01/01/65	06/22/75
281	2*	01/01/55	06/09/75
282	2*	01/01/45	07/01/75
283	1*	07/01/75	07/07/75
286	2*	01/01/55	07/25/75
287	1*	01/01/74	07/30/75
288	1*	01/01/69	07/31/75
289	2	01/01/65	07/31/75
290	2*	01/01/60	07/31/75
291	1	01/01/75	07/29/75
292	2	01/01/72	07/29/75
296	1	01/01/70	08/19/75
297	1*	01/01/71	08/03/75
298	2*	01/01/55	09/17/75
299	1*	01/01/45	09/17/75
301	2	10/01/75	10/23/75
302	2*	01/01/63	10/01/75
304	2*	01/01/70	07/24/74
305	1	10/01/75	10/31/75
306	1*	01/01/59	10/30/74
307	1*	01/01/61	11/24/75
308	2	01/01/74	11/25/75
309	2	01/01/72	11/25/75
311	2*	01/01/66	11/25/75
312	2*	01/01/57	11/25/75
314	1*	01/01/70	12/12/75
315	1	01/01/72	12/12/75

Number	Sex	Date of Birth	Date of Entry
328	2	01/01/76	01/25/76
329	1*	01/01/73	09/14/75
331	2	01/01/68	02/12/76
333	2	01/01/71	02/28/76
335	2*	01/01/60	03/06/76
336	1	01/01/70	03/08/76
339	2	01/01/65	03/12/76
340	2	01/01/73	03/12/76
341	2	01/01/72	03/15/76
342	2	01/01/73	03/15/76
343	1*	01/01/50	03/18/76
345	1*	01/01/70	03/21/76
346	2	01/01/64	03/21/76
349	2	01/01/67	04/05/76
350	2*	01/01/70	04/05/76
351	2*	01/01/66	04/26/76
352	2	01/01/68	04/26/76
354	1	01/01/62	05/22/76
356	2	01/01/76	05/25/76
359	2	01/01/66	06/14/76
360	1	01/01/72	06/19/76
361	2	01/01/74	06/19/76
362	2	01/01/75	06/01/76
364	1	01/01/56	06/01/76
365	1	01/01/51	07/05/76
366	1	01/01/50	08/01/76
367	2	01/01/68	08/16/76
368	2*	01/01/60	08/18/76
369	1*	01/01/74	09/02/76
370	1	01/01/68	09/06/76
372	2	01/01/75	09/09/76
376	2*	01/01/56	10/01/76
377	1*	01/01/73	10/07/76
378	2*	01/01/69	10/07/76
379	1	01/01/72	10/07/76
380	2	01/01/71	10/11/76
381	1*	01/01/69	10/21/76
382	1*	01/01/56	11/02/76
383	2*	01/01/70	11/02/76
384	2	01/01/65	11/02/76
385	2	01/01/73	11/02/76
386	1*	01/01/56	11/02/76
387	1*	01/01/46	11/02/76
388	1*	01/01/66	11/14/76
389	1*	01/01/72	11/25/76
391	2	01/01/68	11/30/76
392	1	01/01/65	11/30/76
393	2*	01/01/57	11/30/76
394	1	01/01/66	12/06/76
395	2	01/01/69	12/06/76
397	2*	01/01/65	12/14/76
399	2	01/01/64	12/21/76

Number	Sex	Date of Birth	Date of Entry
404	2	01/01/71	12/21/76
405	2	01/01/70	01/05/77
407	1*	02/01/77	02/14/77
410	2	01/01/64	03/01/77
411	1*	01/01/72	03/01/77
412	1	01/01/72	03/04/77
413	2	01/01/74	03/04/77
414	2*	01/01/37	03/17/77
415	1	01/01/61	03/23/77
416	1	01/01/76	03/23/77
417	2	01/01/71	03/13/77
418	2*	01/01/60	04/13/77
419	2	01/01/74	04/13/77
423	2	01/01/71	04/21/77
426	2*	01/01/47	04/21/77
427	1	01/01/73	04/26/77
428	2	01/01/71	05/08/77
432	1	01/01/73	05/16/77
434	2	01/01/74	05/16/77
435	1*	01/01/65	05/30/77
436	2	01/01/69	05/30/77
438	2*	01/01/48	06/20/77
439	2*	01/01/62	06/20/77
441	2*	01/01/59	07/03/77
443	1*	01/01/63	07/17/77
444	2*	04/01/75	10/10/76
445	1	01/01/70	01/25/74
446	2*	07/01/77	07/22/77
447	1*	01/01/67	08/01/77
448	1	01/01/67	08/01/77
449	2*	01/01/50	02/18/75
450	1	01/01/72	08/09/77
451	2*	01/01/71	08/09/77
452	2	01/01/64	08/09/77
453	1	01/01/71	08/19/77
454	2*	01/01/42	08/23/77
455	1*	01/01/59	08/23/77
456	1*	01/01/47	09/02/77
458	1*	01/01/52	09/02/77
459	1*	01/01/55	09/02/77
460	1*	01/01/57	09/06/77
461	1*	01/01/67	09/06/77
463	1	01/01/57	09/21/77
464	2	01/01/66	09/26/77
465	2*	01/01/75	09/26/77
468	2	01/01/74	10/10/77
469	2	01/01/64	10/14/77
470	1	01/01/71	10/14/77
473	2	10/01/77	10/22/77
474	2	01/01/67	11/04/77
475	1	01/01/67	11/04/77
477	1*	01/01/63	11/04/77

Number	Sex	Date of Birth	Date of Entry
486	2	01/01/70	01/10/78
489	2	01/01/72	01/20/78
492	1*	01/01/66	01/25/78
493	1*	01/01/58	02/03/78
494	1*	01/01/53	02/14/78
495	2	01/01/66	03/01/78
496	2	01/01/68	03/01/78
497	1*	01/01/68	03/01/78
498	2*	01/01/76	03/07/78
499	2	01/01/64	03/08/78
500	2	01/01/75	03/15/78
501	1	01/01/63	03/17/78
502	2*	01/01/64	03/17/78
503	2	01/01/58	03/28/78
504	1	01/01/63	03/31/78
505	2*	01/01/72	03/31/78
507	2*	01/01/62	04/03/78
508	2	01/01/56	04/09/78
511	1	01/01/65	04/16/78
512	1*	01/01/65	04/19/78
513	2	01/01/74	04/28/74
515	2	05/01/78	05/06/78
517	1*	01/01/52	05/08/78
518	1*	01/01/47	05/08/78
519	1*	01/01/43	05/10/78
520	2*	01/01/43	05/10/78
522	1*	01/01/48	05/15/78
524	2*	01/01/51	05/15/78
527	2*	01/01/48	05/16/78
529	1	01/01/77	05/23/78
530	2	01/01/62	05/23/78
532	2*	01/01/63	06/08/78
533	2	01/01/72	06/12/72
534	1*	01/01/73	06/12/78
535	2	01/01/66	06/12/78
535	2	01/01/66	06/12/78
536	2	01/01/67	06/12/78
538	2*	01/01/58	06/21/78
539	2*	01/01/58	06/21/78
542	2	01/01/58	06/28/78
544	1	01/01/62	07/27/78
546	1*	01/01/68	08/01/78
547	2*	01/01/48	08/04/78
548	1*	01/01/72	08/08/78
550	1	01/01/63	08/18/78
551	2	01/01/75	08/21/78
552	2*	01/01/62	08/21/78
553	1*	01/01/72	08/21/78
555	2*	01/01/73	09/05/78
557	1	01/01/74	09/09/78
558	2	01/01/69	09/09/78
559	2	01/01/73	09/14/78

Number	Sex	Date of Birth	Date of Entry
565	2	01/01/56	09/27/78
566	1	01/01/67	09/27/78
567	1	01/01/77	09/27/78
568	1	01/01/77	09/27/78
570	2	01/01/67	10/02/78
571	2	01/01/48	10/02/78
572	2*	01/01/63	10/15/78
573	2*	01/01/71	10/15/78
574	1	01/01/70	10/22/78
575	1	01/01/66	10/22/78
577	2*	01/01/62	10/22/78
578	2	01/01/68	10/22/78
581	1	01/01/74	10/25/78
582	1	04/01/78	11/01/78
583	2	01/01/70	11/01/78
584	2	01/01/66	11/01/78
585	2*	01/01/65	11/01/78
586	2	01/01/69	11/06/78
587	1*	01/01/72	11/06/78
588	2	01/01/69	11/06/78
589	1*	01/01/65	11/07/78
594	1	05/01/78	11/22/78
595	2	01/01/72	11/29/78
596	2*	01/01/58	11/21/78
597	1	01/01/76	11/21/78
598	2*	01/01/73	12/10/78
599	2	01/01/74	12/10/78
600	2	01/01/75	12/10/78
602	1*	01/01/66	12/10/78
603	2	01/01/66	12/10/78
604	1*	01/01/58	12/10/78
606	2*	01/01/70	01/12/79
607	2	01/13/79	01/13/79
608	1*	01/01/77	01/16/79
609	1*	01/01/69	01/22/79
610	2*	01/01/56	02/09/79
611	1	01/01/74	02/09/79
612	1	01/01/62	02/09/79
613	2*	01/01/59	02/09/79
615	2*	01/01/62	02/09/79
616	2	01/01/69	02/15/69
617	2*	01/01/49	02/15/79
619	2	01/01/59	02/20/79
620	1	01/01/61	02/21/79
622	1	01/01/72	02/25/79
623	2	01/01/70	03/03/79
624	1*	01/01/69	03/03/79
625	1*	01/01/69	03/03/79
626	2*	03/01/79	03/02/79
627	2*	01/01/62	03/18/79
629	2	01/01/69	03/19/79
630	2	01/01/68	03/19/79

Number	Sex	Date of Birth	Date of Entry
636	2	01/01/69	04/02/79
638	2	01/01/76	04/05/79
639	2*	01/01/71	04/05/79
640	2	01/01/65	04/05/79
642	2	05/01/77	04/18/79
644	2*	01/01/49	04/18/79
645	2*	01/01/73	04/18/79
646	2	01/01/64	04/20/79
648	1	01/01/65	05/02/79
649	2	01/01/63	05/02/79
650	1	05/01/79	05/10/79
651	1*	05/01/79	05/12/79
654	2*	01/01/39	05/17/79
655	2	05/01/79	05/19/79
659	1*	01/01/65	06/06/79
661	2	01/01/72	06/15/79
662	2	01/01/74	06/15/79
664	2*	01/01/61	06/15/79
667	2	01/01/69	06/22/79
668	2	01/01/75	06/22/79
669	2	01/01/54	06/22/79
670	1*	01/01/70	06/22/79
673	1*	01/01/59	06/27/79
675	2*	01/01/49	07/17/79
679	1	01/01/59	07/19/79
682	1*	01/01/63	07/31/79
684	2	01/01/72	08/20/79
685	2*	01/01/72	08/21/79
688	2*	01/01/49	09/11/79
689	2*	01/01/61	09/11/79
690	2*	05/01/76	09/11/79
692	1	01/01/69	09/14/79
693	2*	01/01/68	09/14/79
694	1	01/01/76	09/14/79
696	1	01/01/77	09/21/79
698	1	01/01/75	09/21/79
699	2	01/01/63	09/25/79
700	1	01/01/70	09/25/79
704	2*	01/01/74	09/26/79
705	1*	01/01/59	09/26/79
706	2	01/01/61	09/26/79
707	1*	01/01/39	09/28/79
708	1*	01/01/63	09/28/79
709	1	03/01/79	10/12/79
711	1*	01/01/64	11/06/79
712	2*	01/01/69	11/06/79
713	2	01/01/74	11/27/79
714	2	01/01/63	12/11/79
716	1	01/01/67	12/11/79
718	2*	01/01/65	12/11/79
719	2	01/01/65	12/11/79
720	2*	01/01/49	12/11/79

Number	Sex	Date of Birth	Date of Entry
726	1	01/01/60	01/08/80
727	1	01/01/75	01/10/80
728	2	01/01/70	01/24/80
729	2	01/01/79	01/24/80
731	1	01/01/75	01/24/80
732	1*	01/01/77	01/24/80
737	2*	01/01/69	02/07/80
738	2	01/01/75	02/07/80
739	2	01/01/76	02/07/76
740	2	01/01/72	02/07/80
741	2	01/01/71	02/07/80
742	1*	01/01/72	02/07/80
743	1*	01/01/58	02/07/80
744	2	01/01/76	02/11/80
745	1*	01/01/63	02/11/80
746	2	01/01/60	02/12/80
750	2*	01/01/45	02/29/80
751	2	01/01/72	02/29/80
753	1*	01/01/50	03/04/80
754	2	01/01/76	03/04/80
755	2*	01/01/66	03/04/80
756	2	01/01/74	03/11/80
758	2*	01/01/62	03/11/80
761	2	01/01/70	03/10/80
762	2	01/01/73	03/18/80
763	2	01/01/74	03/18/80
765	2*	01/01/50	03/27/80
766	2	01/01/65	03/27/80
767	2*	01/01/55	03/27/80
768	2	03/01/80	03/29/80
769	2	01/01/73	04/08/80
770	2	01/01/70	04/08/80
773	2	01/01/71	04/17/80
775	2*	01/01/68	04/18/80
777	2*	01/01/78	04/23/80
780	2	01/01/77	04/24/80
781	2	01/01/69	04/24/80
783	2*	01/01/70	04/24/80
784	2	01/01/69	04/25/80
785	2	01/01/73	04/25/80
786	2	01/01/74	04/25/80
787	2	01/01/55	04/28/80
788	2	01/01/76	04/28/80
791	2	01/01/76	04/30/80
792	2	01/01/75	05/09/80
793	2	01/01/73	05/09/80
794	1*	01/01/73	05/30/80
797	1	01/01/71	06/04/80
798	1*	01/01/72	06/07/80
801	1	01/01/66	06/17/80
802	2	05/01/69	06/17/80
807	2	01/01/67	06/26/80

Number	Sex	Date of Birth	Date of Entry
811	1	01/01/73	07/03/80
812	1	01/01/60	07/03/80
813	1*	01/01/65	07/03/80
814	2*	01/01/76	07/08/80
816	1	01/01/68	07/11/80
818	1*	01/01/70	07/14/80
819	1	01/01/62	07/25/80
822	2*	01/01/73	08/01/80
823	2*	01/01/55	08/01/80
825	2	01/01/70	08/05/80
827	1*	01/01/60	08/20/80
828	1	01/01/75	08/21/80
829	1	01/01/72	09/02/80
830	2	01/01/76	09/06/80
832	2	01/01/76	09/06/80
833	1*	01/01/70	09/06/80
834	2*	09/01/80	09/08/80
836	2*	01/01/70	09/17/80
838	2	01/01/77	09/17/80
839	2	01/01/78	09/17/80
840	2	01/01/71	09/19/80
842	1	01/01/77	09/24/80
843	2	01/01/69	09/24/80
844	2*	01/01/55	09/24/80
846	2	01/01/68	10/01/80
847	1*	01/01/55	10/01/80
848	2	01/01/75	10/01/80
849	2	01/01/75	10/01/80
850	1*	01/01/56	10/01/80
851	2	01/01/65	10/03/80
852	2*	01/01/62	10/03/80
857	1*	01/01/73	10/08/80
858	2	01/01/75	10/08/80
864	1	06/01/69	10/12/80
865	2	01/01/70	10/12/80
866	2	01/01/62	10/13/80
867	2	01/01/73	10/13/80
869	2	01/01/73	10/29/80
870	2	01/01/64	10/31/80
871	2*	01/01/60	10/31/80
872	1	01/01/65	10/31/80
873	2	01/01/78	11/04/80
875	1	01/01/65	11/06/80
877	1	01/01/72	11/06/80
878	2	01/01/70	11/06/80
879	2	01/01/73	11/07/80
880	2	01/01/73	11/07/80
881	1	05/01/77	11/07/80
882	2*	01/01/68	11/18/80
884	2	01/01/74	11/18/80
887	1	01/01/73	11/19/80
892	2	01/01/70	11/21/80

Number	Sex	Date of Birth	Date of Entry
897	2	01/01/72	11/29/80
898	2	01/01/75	11/28/80
901	2	01/01/68	12/01/80
904	1	01/01/71	12/04/80
905	1	01/01/72	12/04/80
906	1*	01/01/63	12/04/80
907	2*	01/01/50	12/04/80
909	1*	01/01/74	12/09/80
910	1	01/01/60	12/10/80
911	1	01/01/70	12/10/80
913	2	01/01/77	12/12/80
916	1	01/01/73	12/16/80
917	1	01/01/77	12/15/80
918	2	01/01/73	12/15/80
920	1*	01/01/65	12/18/80
925	2	01/01/79	01/03/81
926	1	01/01/66	01/14/81
930	1	01/01/70	01/16/81
931	2*	01/01/72	01/20/81
932	2	01/01/76	01/20/81
933	1*	01/01/71	01/22/81
935	1	01/01/69	02/04/81
936	2	06/01/75	02/04/81
938	2*	01/01/79	02/14/81
939	2	01/01/63	02/14/81
940	2	09/01/80	02/14/81
943	2*	01/01/77	03/03/81
944	1	01/01/56	03/10/81
947	1	01/01/74	03/11/81
948	2	01/01/64	03/11/81
949	2	01/01/74	03/11/81
950	1	01/01/73	03/11/81
951	2	01/01/76	03/16/81
952	2	01/01/72	03/16/81
953	2	01/01/58	03/20/81
954	1	01/01/73	03/20/81
955	2*	01/01/60	03/20/81
956	1*	01/01/73	03/20/81
959	2	01/01/66	03/24/81
960	2*	01/01/41	03/24/81
964	2	01/01/76	03/30/81
965	1	01/01/50	03/31/81
966	2	01/01/70	04/03/81
967	1	01/01/75	04/03/81
968	2*	01/01/65	04/03/81
969	1	01/01/73	04/03/81
970	1*	01/01/73	04/06/81
971	1*	01/01/71	04/06/81
972	2*	01/01/73	04/06/81
973	2	01/01/71	04/06/81
974	1	01/01/72	04/07/81
975	1	01/01/78	04/05/81

Number	Sex	Date of Birth	Date of Entry
982	1*	01/01/50	04/16/81
984	2	01/01/61	04/22/81
985	2*	01/01/64	04/22/81
986	2*	01/01/56	04/24/81
987	2	06/01/76	04/24/81
988	2	01/01/63	04/24/81
989	2	01/01/60	04/24/81
990	2	01/01/61	04/24/81
994	2	01/01/64	04/30/81
995	2	01/01/76	04/30/81
996	2	01/01/79	04/30/81
997	2	01/01/63	04/30/81
998	2	01/01/62	04/30/81
999	2	01/01/76	05/01/81
1000	2*	01/01/70	05/01/81
1001	2*	01/01/70	05/05/81
1002	2	04/01/72	05/07/81
1003	1	01/01/65	05/07/81
1004	2	08/01/67	05/12/81
1005	1	04/01/68	05/12/81
1006	2	01/01/75	05/12/81
1007	2*	01/01/61	05/12/81
1008	2*	01/01/59	05/15/81
1009	2*	01/01/56	05/15/81
1010	2	01/01/75	05/14/81
1011	2	01/01/78	05/14/81
1012	1	01/01/67	05/14/81
1015	2*	01/01/71	05/20/81
1016	2	01/01/76	05/20/81
1019	2*	05/01/81	05/23/81
1020	1*	01/01/60	05/26/81
1021	2*	01/01/60	05/26/81
1023	2*	01/01/71	05/29/81
1024	2	01/01/72	05/29/81
1027	2	01/01/74	06/08/81
1029	2	01/01/74	06/09/81
1030	1	05/01/67	06/10/81
1031	2	01/01/66	06/16/81
1033	1	01/01/66	06/30/81
1034	2	01/01/76	06/30/81
1036	1*	08/01/79	06/30/81
1037	1	07/01/78	06/30/81
1038	2	01/01/68	06/30/81
1039	2	01/01/70	07/07/81
1040	2	01/01/69	07/07/81
1041	2	01/01/69	07/09/81
1042	2	01/01/71	07/09/81
1043	2	01/01/72	07/09/81
1044	2	01/01/70	07/09/81
1045	2*	01/01/72	07/09/81
1046	2	01/01/76	07/09/81
1047	2	01/01/76	07/09/81

Number	Sex	Date of Birth	Date of Entry
1051	1*	01/01/75	07/10/81
1053	1	01/01/62	07/14/81
1054	2	01/01/65	07/14/81
1055	2	01/01/81	07/14/81
1057	2	01/01/76	07/16/81
1058	2	01/01/75	07/17/81
1059	2*	01/01/71	07/17/81
1060	1	01/01/71	07/20/81
1061	2*	01/01/71	07/20/81
1062	2	01/01/71	07/28/81
1063	1	01/01/70	07/28/81
1065	1*	01/01/74	08/10/81
1068	1*	01/01/47	08/25/81
1069	1*	01/01/75	08/27/81
1071	2	01/01/68	08/27/81
1072	1	01/01/71	08/27/81
1073	2	01/01/73	09/04/81
1074	2	01/01/54	09/04/81
1076	2*	01/01/66	09/15/81
1077	1*	01/01/72	09/15/81
1078	2*	01/01/75	09/15/81
1079	1*	01/01/72	09/15/81
1080	2	01/01/65	09/22/81
1081	1	01/01/65	09/22/81
1082	2*	01/01/56	09/22/81
1083	2*	01/01/60	09/22/81
1085	1	08/01/72	09/22/81
1088	2*	01/01/69	09/25/81
1089	1*	01/01/56	09/25/81
1090	2*	01/01/56	10/08/81
1091	2	01/01/76	10/08/81
1094	2	01/01/79	10/13/81
1097	2	01/01/70	10/14/81
1100	2	01/01/75	10/14/81
1101	2	01/01/76	10/14/81
1102	2	01/01/72	10/14/81
1104	2	01/01/62	10/14/82
1105	2	01/01/76	10/14/81
1106	2*	01/01/51	10/16/81
1107	1	02/01/80	10/20/81
1108	2	01/01/56	10/20/81
1109	1	01/01/71	10/20/81
1111	1*	01/01/69	10/23/81
1112	1	01/01/69	10/23/81
1113	1	01/01/71	10/23/81
1115	2	01/01/72	10/27/81
1116	2	01/01/62	10/27/81
1118	1	01/01/76	10/30/81
1121	2	01/01/67	11/05/81
1122	2	05/01/79	11/04/81
1123	2	01/01/70	11/06/81
1124	2*	01/01/62	11/06/81

Number	Sex	Date of Birth	Date of Entry
1129	1*	06/01/75	11/17/81
1131	1	06/01/79	11/24/81
1132	2	01/01/73	11/25/81
1133	2	01/01/79	11/25/81
1135	2*	01/01/65	11/30/81
1136	1*	01/01/71	12/04/81
1137	1	01/01/78	12/04/81
1138	1	01/01/77	12/22/81
1141	2*	01/01/50	12/16/81
1142	1*	01/01/61	12/30/81
1145	2*	01/01/71	01/06/82
1148	1*	01/01/57	01/07/82
1149	2	01/01/68	01/07/82
1150	2	01/01/75	01/07/82
1151	2*	01/01/72	01/07/82
1153	1	01/01/75	01/20/82
1154	2	01/01/79	01/20/82
1155	2	01/01/77	01/20/82
1156	1	01/01/77	01/20/82
1158	1	01/01/80	01/20/82
1159	2	01/01/77	01/21/82
1160	1	01/01/77	01/21/82
1161	2*	01/01/81	01/26/81
1162	2*	01/01/78	02/26/82
1163	2	01/01/67	01/21/82
1164	2	01/01/62	02/21/82
1165	1	01/01/64	01/21/82
1168	2	01/01/74	01/28/82
1169	1	01/01/79	01/28/82
1175	1	01/01/75	02/05/82
1176	2	01/01/77	02/05/82
1177	1	01/01/64	02/08/82
1178	2*	01/01/65	12/08/82
1179	2	01/01/62	02/08/82
1180	1	01/01/81	02/15/82
1181	1*	01/01/57	02/16/82
1182	2*	01/01/55	02/19/82
1183	1	01/01/52	02/19/82
1184	1	02/01/82	02/01/82
1185	2*	01/01/66	02/23/82
1186	1	01/01/75	02/23/82
1187	2	01/01/74	02/23/82
1188	2	01/01/71	02/25/82
1191	2*	01/01/75	03/04/82
1195	2	01/01/74	03/08/82
1196	2*	01/01/73	03/08/82
1197	2	01/01/72	03/08/82
1198	2*	01/01/70	03/08/82
1199	2	01/01/72	03/08/82
1200	1	01/01/72	03/10/82
1201	2	01/01/62	03/12/82
1202	2*	01/01/67	03/12/82

Number	Sex	Date of Birth	Date of Entry
1218	2*	01/01/60	03/24/82
1225	2	01/01/72	04/02/82
1226	2	01/01/78	04/02/82
1227	2	01/01/77	04/02/82
1229	1	01/01/81	04/02/82
1230	2	01/01/72	04/02/82
1231	1	04/01/82	04/02/82
1232	2*	04/01/82	04/02/82
1234	2*	01/01/67	04/06/82
1238	1	01/01/75	04/22/82
1239	2	01/01/73	04/22/82
1240	1*	01/01/48	04/22/82
1241	2	01/01/74	04/22/82
1243	2*	01/01/76	04/26/82
1245	2	01/01/77	04/27/82
1246	2	01/01/78	04/27/82
1247	1	06/01/80	04/27/82
1248	1	01/01/72	04/27/82
1249	2	01/01/75	04/27/82
1251	2*	01/01/53	05/07/82
1252	2	01/01/60	05/07/82
1256	2	01/01/75	05/18/82
1258	1	01/01/77	05/18/82
1259	1	01/01/58	05/21/82
1260	2*	01/01/65	05/21/82
1262	2	01/01/68	05/25/82
1263	1	01/01/68	05/25/82
1264	2	01/01/57	05/27/82
1265	1	01/01/62	05/27/82
1266	2	01/01/67	05/28/82
1270	1*	01/01/57	06/01/82
1271	2	01/01/62	06/01/82
1272	2*	01/01/75	06/01/82
1274	1	01/01/62	06/09/82
1275	1	01/01/79	06/11/82
1276	2	01/01/67	06/11/82
1279	2	01/01/75	06/16/82
1280	2	01/01/57	06/16/82
1281	2	01/01/66	06/18/82
1282	2	05/01/71	06/18/82
1283	2	08/01/71	06/18/82
1284	2	11/01/72	06/18/82
1285	2	04/01/76	06/18/82
1286	1	10/01/73	06/18/82
1287	1*	09/01/74	06/18/82
1288	1	06/01/75	06/18/82
1290	1*	01/01/57	06/25/82
1293	2	01/01/50	07/07/82
1295	2*	01/01/79	07/07/82
1297	2*	05/01/82	07/07/82
1298	2	01/01/78	07/07/82
1299	2*	01/01/40	07/07/82

Number	Sex	Date of Birth	Date of Entry
1308	2*	06/01/72	07/21/82
1309	2	05/01/77	07/21/82
1311	1	01/01/75	07/22/82
1319	2	01/01/70	08/02/82
1320	1	01/01/68	08/03/82
1321	2*	01/01/63	08/03/82
1322	1	01/01/60	08/03/82
1323	1	01/01/60	08/03/82
1324	2	01/01/70	08/06/82
1326	2	01/01/72	08/06/82
1331	1	01/01/72	08/12/82
1333	1	01/01/76	09/02/82
1334	2	01/01/76	09/02/82
1341	1	01/01/72	09/17/82
1342	1*	01/01/68	09/17/82
1344	1*	01/01/81	09/17/82
1345	1	01/01/65	09/17/82
1346	1*	01/01/69	09/21/82
1347	1	01/01/79	09/21/82
1349	1	01/01/80	10/05/82
1350	1	01/01/64	10/08/82
1353	2*	01/01/68	10/08/82
1360	1*	01/01/70	10/27/82
1361	1	01/01/72	10/27/82
1371	1	01/01/70	11/10/82
1372	1	01/01/45	11/12/82
1376	1	01/01/74	11/17/82
1377	2	01/01/80	11/17/82
1378	2	01/01/76	11/17/82
1385	1*	01/01/72	11/18/82
1388	1	01/01/72	11/18/82
1390	1	01/01/72	11/19/82
1391	1*	01/01/70	11/19/82
1397	2*	01/01/69	12/08/82
1398	2*	01/01/68	12/09/82
1399	2*	01/01/69	12/09/82
1400	2*	01/01/42	12/09/82
1401	2*	01/01/68	12/09/82
1402	2	01/01/70	12/11/82
1403	2	01/01/76	12/11/82
1404	1	01/01/81	12/11/82
1411	2	01/01/62	12/21/82
1412	1	01/01/72	12/21/82
1413	2	01/01/70	12/21/82
1414	2*	01/01/69	12/21/82
1416	1	01/01/77	12/21/82
1419	2	01/01/75	01/07/83
1422	1*	01/01/63	01/10/83
1424	1	01/01/53	01/11/83
1426	2	01/01/58	01/19/83
1427	1	01/01/50	01/19/83
1428	2	01/01/80	01/18/83

Number	Sex	Date of Birth	Date of Entry
1438	2	01/01/70	02/04/83
1441	2*	01/01/63	02/09/83
1442	2*	01/01/74	02/09/83
1443	2	01/01/64	02/09/83
1444	2	01/01/64	02/09/83
1445	2	01/01/67	02/15/83
1447	1*	01/01/68	02/15/83
1448	2*	01/01/63	02/15/83
1449	2	01/01/61	02/15/83
1450	1	01/01/67	02/16/83
1451	2	01/01/63	02/18/83
1452	2	01/01/68	02/21/83
1453	2	01/01/74	02/21/83
1454	2*	01/01/71	02/21/83
1455	2	01/01/72	02/22/83
1456	1	01/01/77	02/22/83
1457	1	01/01/76	02/22/83
1458	1	10/01/81	02/22/83
1461	2*	01/01/63	02/24/83
1465	1	01/01/55	03/10/83
1466	1	01/01/63	03/10/83
1467	2*	01/01/70	03/10/83
1470	2	01/01/63	03/13/83
1471	2*	01/01/53	03/13/83
1472	2	01/01/53	03/13/83
1473	2	01/01/76	03/16/83
1475	1*	01/01/79	03/16/83
1476	2	01/01/59	03/16/83
1480	1*	06/30/82	04/01/83
1483	1*	01/01/53	04/06/83
1487	1	01/01/71	04/12/83
1488	2	01/01/69	04/12/83
1490	2	01/01/80	04/12/83
1492	2	01/01/76	04/12/83
1494	2	01/01/53	04/12/83
1496	2	01/01/72	04/15/83
1497	2	01/01/78	04/15/83
1498	1	01/01/80	04/15/83
1499	1	11/01/81	04/15/83
1500	2	01/01/72	04/19/83
1501	2*	01/01/67	04/19/83
1502	1	01/01/73	04/19/83
1503	1	01/01/66	04/20/83
1504	2*	01/01/63	04/20/83
1505	2	01/01/68	04/20/83
1506	2	01/01/70	04/25/83
1507	2*	01/01/73	04/25/83
1511	2*	01/01/73	04/28/83
1514	1*	01/01/58	05/05/83
1515	2	01/01/58	05/05/83
1516	2	01/01/58	05/05/83
1518	2	01/01/71	05/10/83

Number	Sex	Date of Birth	Date of Entry
1528	1	01/01/70	05/13/83
1529	2*	01/01/63	05/13/83
1530	2	01/01/59	05/13/83
1533	2	01/01/72	05/18/83
1536	2*	01/01/48	05/25/83
1537	2	01/01/74	05/25/83
1538	1	01/01/63	05/25/83
1539	2*	01/01/43	05/25/83
1540	2	01/01/68	05/25/83
1541	1*	01/01/60	05/25/83
1546	2*	01/01/63	06/10/83
1547	2*	01/01/73	06/10/83
1548	2	06/11/83	06/11/83
1552	2	01/01/63	06/22/83
1554	2	01/01/50	06/30/83
1555	2*	01/01/66	06/30/83
1557	1	01/01/58	06/30/83
1562	1	01/01/50	07/11/83
1564	2*	01/01/63	07/12/83
1565	1	01/01/63	07/12/83
1566	2	01/01/75	07/12/83
1568	1*	01/01/79	07/12/83
1569	2	01/01/78	07/12/83
1571	2	01/01/81	07/12/83
1572	1	01/01/72	07/15/83
1573	2	01/01/73	07/15/83
1574	2*	01/01/58	07/15/83
1576	2	01/01/78	07/15/83
1579	2	01/01/75	07/19/83
1580	2	01/01/71	07/19/83
1581	2	01/01/76	07/21/83
1584	2	06/01/74	07/21/83
1585	2	05/01/73	07/21/83
1586	2	01/01/67	07/21/83
1587	1	01/01/73	07/27/83
1588	2	01/01/76	07/27/83
1590	2	01/01/73	08/01/83
1591	1*	01/01/68	08/01/83
1592	2	01/01/63	08/01/83
1593	1	01/01/57	08/08/83
1594	1	01/01/68	08/08/83
1595	2	04/01/77	08/11/83
1596	2	01/01/70	08/16/83
1597	1	01/01/77	08/16/83
1598	2	01/01/78	08/16/83
1600	2	01/01/79	08/19/83
1603	1	01/01/72	08/19/83
1604	2	01/01/70	08/19/83
1608	2	01/01/63	08/24/83
1609	2	01/01/71	08/24/83
1612	1	01/01/73	08/26/83
1613	1	01/01/78	08/26/83

Number	Sex	Date of Birth	Date of Entry
1622	2*	01/01/79	09/08/83
1623	2	01/01/75	09/08/83
1628	1	01/01/70	09/16/83
1629	1	01/01/65	09/16/83
1631	2	01/01/70	09/21/83
1632	1	01/01/74	09/21/83
1633	1	01/01/73	09/23/83
1634	2	01/01/75	10/03/83
1638	1	01/01/60	10/07/83
1639	2	01/01/67	10/07/83
1640	1	01/01/67	10/07/83
1649	1	02/01/76	10/17/83
1650	1	02/01/78	01/17/83
1651	2	07/01/69	01/17/83
1658	2	01/01/70	10/21/83
1659	2*	01/01/78	10/21/83
1660	2*	01/01/70	10/25/83
1661	2	06/01/74	10/25/83
1664	1	01/01/58	10/27/83
1665	1*	01/01/53	10/28/83
1666	1	01/01/58	10/28/83
1668	2*	01/01/63	11/01/83
1671	2*	01/01/70	11/01/83
1672	1	01/01/76	11/01/83
1674	1	01/01/67	11/04/83
1675	1	01/01/60	11/04/83
1676	2	01/01/58	11/04/83
1677	1	01/01/75	11/04/83
1680	2*	01/01/43	11/10/83
1681	1	01/01/66	11/10/83
1682	1	01/01/66	11/10/83
1684	1	01/01/63	11/17/83
1688	2	01/01/63	11/21/83
1689	1	01/01/71	11/21/83
1690	1	01/01/76	11/23/83
1691	1*	01/01/70	11/28/83
1694	2*	01/01/71	12/01/83
1696	2	06/01/82	12/01/83
1697	2	01/01/72	12/01/83
1699	2	01/01/75	12/01/83
1700	1	01/01/75	12/01/83
1703	2*	01/01/72	12/09/83
1707	1	01/01/80	12/09/83
1709	1	01/01/73	12/09/83
1712	1	01/01/81	12/16/83
1714	2	01/01/79	12/19/83
1716	1	01/01/76	12/22/83
1717	2	01/01/71	12/22/83
1718	1*	01/01/68	01/06/84
1721	2	01/01/79	01/10/84
1723	2	01/01/64	01/13/84
1724	2	08/01/71	01/13/84

Number	Sex	Date of Birth	Date of Entry
1733	1	01/01/79	01/21/84
1736	2	01/01/72	02/08/84
1738	1	01/01/72	02/16/84
1739	1	01/01/61	02/16/84
1740	2	01/01/74	02/16/84
1741	2	01/01/77	02/16/84
1742	2	01/01/58	02/16/84
1743	2	01/01/72	02/03/84
1744	2	01/01/80	02/03/84
1745	2	01/01/78	02/03/84
1746	1	01/01/83	02/06/84
1747	2*	01/01/82	02/10/84
1749	1	01/01/73	02/10/84
1753	2	01/01/70	02/21/84
1754	1	01/01/73	02/21/84
1758	2*	01/01/54	02/28/84
1759	2	01/01/64	02/28/84
1760	2	01/01/79	02/28/84
1763	2	01/01/80	03/09/84
1764	2	01/01/78	03/13/84
1766	2	01/01/72	03/13/84
1767	2	01/01/77	03/13/84
1768	1*	01/01/71	03/14/84
1772	2	04/01/70	03/20/84
1773	2	01/01/59	03/20/84
1774	2	01/01/62	03/20/84
1776	2*	01/01/68	03/20/84
1782	2	01/01/75	03/23/84
1783	1	01/01/70	03/29/84
1784	1*	01/01/71	03/29/84
1785	2	01/01/75	04/05/84
1786	2	01/01/73	04/05/84
1788	2	01/01/66	04/05/84
1790	1	04/01/73	04/05/84
1791	2	04/01/84	04/07/84
1793	1	01/01/83	04/12/84
1794	1	01/01/83	04/12/84
1795	2	01/01/83	04/12/84
1797	2	01/01/74	04/12/84
1799	2	04/01/84	04/18/84
1806	2*	07/01/71	04/25/84
1807	1	06/01/72	04/25/84
1808	2	08/01/74	04/25/84
1810	1*	01/01/50	04/27/84
1811	1	01/01/68	04/27/84
1814	2	05/01/84	05/02/84
1818	2*	01/01/78	05/09/84
1822	1*	01/01/39	05/11/84
1823	2	01/01/76	05/11/84
1824	1	01/01/64	05/11/84
1825	2	01/01/70	05/11/84
1828	2	01/01/77	05/16/84

Number	Sex	Date of Birth	Date of Entry
1832	2	01/01/60	05/18/84
1833	1	01/01/60	05/23/84
1834	1	01/01/60	05/23/84
1835	1*	01/01/79	05/23/84
1836	2*	01/01/79	05/23/84
1837	1	01/01/60	05/25/84
1840	2	01/01/59	05/25/84
1841	1	01/01/73	05/25/84
1843	1	01/01/60	05/30/84
1844	2	01/01/69	05/30/84
1845	2	01/01/80	06/07/84
1846	1	01/01/80	06/07/84
1847	2	01/01/67	06/09/84
1848	2	01/01/59	06/12/84
1849	1	01/01/78	06/12/84
1850	2	01/01/79	06/12/84
1851	2*	01/01/49	06/14/84
1854	1	01/01/80	06/22/84
1857	1	01/01/54	06/22/84
1858	1	01/01/77	06/22/84
1859	2	01/01/68	06/22/84
1860	2	01/01/69	06/26/84
1861	2	01/01/69	06/26/84
1865	1	01/01/79	07/03/84
1866	1	01/01/79	07/03/84
1867	1	01/01/78	07/03/84
1868	1	01/01/76	07/03/84
1869	2	01/01/77	07/03/84
1870	2	01/01/74	07/03/84
1872	1*	01/01/77	07/06/84
1873	2*	01/01/70	07/06/84
1874	2	01/01/70	07/06/84
1875	2	01/01/80	07/06/84
1876	1	01/01/50	07/06/84
1877	1	01/01/65	07/14/84
1878	1	01/01/66	07/09/84
1879	1	01/01/72	07/11/84
1880	2	01/01/78	07/11/84
1881	2	01/01/72	07/11/84
1884	2	01/01/69	07/17/84
1885	1	01/01/76	07/17/84
1886	1	01/01/76	07/20/84
1887	1	01/01/76	07/20/84
1888	1	01/01/65	07/20/84
1889	2	01/01/71	07/20/84
1890	1	01/01/76	07/20/84
1891	2*	01/01/70	07/20/84
1904	1	01/01/72	08/03/84
1906	2	01/01/68	08/15/84
1907	2	06/01/74	08/15/84
1908	2	06/01/75	08/15/84
1909	2	01/01/64	08/15/84

Number	Sex	Date of Birth	Date of Entry
1915	2*	01/01/64	08/30/84
1916	1	01/01/54	08/30/84
1917	2	01/01/78	09/05/84
1921	2	01/01/75	09/06/84
1922	2*	01/01/80	09/06/84
1923	1	01/01/73	09/06/84
1925	2	01/01/69	09/11/84
1926	1	07/01/82	09/14/84
1927	2*	01/01/50	09/14/84
1928	2*	01/01/64	09/14/84
1936	1	01/01/60	09/19/84
1937	2	01/01/54	09/19/84
1938	1*	01/01/69	09/19/84
1939	2	01/01/60	09/21/84
1945	1*	01/01/60	10/03/84
1946	1	01/01/60	10/03/84
1948	2	01/01/54	10/05/84
1949	2	01/01/64	10/05/84
1952	2	01/01/79	10/12/84
1959	2	01/01/63	10/19/84
1960	2	01/01/59	10/19/84
1961	1	01/01/64	10/19/84
1963	1	01/01/60	10/29/84
1965	1	01/01/44	10/25/84
1966	2*	01/01/59	10/25/84
1967	1*	01/01/60	10/25/84
1968	2	01/01/80	10/25/84
1969	2	01/01/59	10/25/84
1973	1	01/01/59	10/29/84
1976	2	01/01/62	11/06/84
1977	2	01/01/62	11/06/84
1979	1*	01/01/68	11/06/84
1981	1*	01/01/70	11/09/84
1982	2*	01/01/70	11/09/84
1983	2	01/01/75	11/09/84
1984	1	01/01/60	11/09/84
1985	1*	01/01/71	11/09/84
1986	2	11/01/81	11/07/84
1987	2*	01/01/64	11/13/84
1989	1	01/01/73	11/21/84
1990	1	01/01/79	11/21/84
1991	2	01/01/76	11/21/84
1992	2	01/01/73	11/21/84
1993	2*	01/01/67	11/21/84
1995	2*	01/01/70	11/21/84
1997	2	01/01/74	11/27/84
1999	1	01/01/75	11/30/84
2003	2*	01/01/74	12/07/84
2004	1	01/01/79	12/07/84
2005	2*	01/01/69	12/12/84
2007	1	01/01/81	12/12/84
2010	2*	01/01/61	12/14/84

Number	Sex	Date of Birth	Date of Entry
2014	1	01/01/72	12/14/84
2015	1	01/01/72	12/14/84
2016	2	01/01/69	12/14/84
2017	2*	01/01/70	12/11/84
2025	2*	01/01/70	12/18/84
2027	2*	01/01/64	12/21/84
2031	1*	01/01/81	01/10/85
2042	2	01/01/45	01/22/85
2043	2*	01/01/60	01/22/85
2044	2*	01/01/82	01/24/85
2047	2	01/01/70	01/29/85
2048	1	07/01/75	01/29/85
2050	2*	01/01/81	02/01/85
2054	1*	05/01/72	02/05/85
2055	2*	06/01/69	02/05/85
2056	2	01/01/62	02/05/85
2059	2*	01/01/57	02/07/85
2060	2	01/01/50	02/07/85
2061	1*	01/01/67	02/07/85
2063	2*	01/01/60	02/07/85
2066	1	01/01/70	02/08/85
2067	1*	01/01/70	02/08/85
2068	2	01/01/70	02/08/85
2069	1	01/01/68	02/13/85
2073	2	01/01/74	02/18/85
2074	1	01/01/76	02/18/85
2075	1	01/01/78	02/18/85
2076	1	01/01/79	02/18/85
2081	2	01/01/70	02/21/85
2082	1	01/01/75	02/21/85
2085	1	01/01/83	02/28/85
2086	1*	01/01/65	03/06/85
2093	2*	01/01/62	03/08/85
2099	2	01/01/73	03/08/85
2100	1	01/01/71	03/08/85
2101	2	01/01/73	03/13/85
2102	2	01/01/79	03/13/85
2104	2	01/01/67	03/15/85
2105	2	01/01/65	03/15/85
2106	2	01/01/78	03/15/85
2107	2	01/01/74	03/15/85
2108	2	01/01/72	03/15/85
2114	2	01/01/55	03/19/85
2115	2*	01/01/70	03/19/85
2116	1	01/01/66	03/19/85
2117	2	01/01/75	03/21/85
2118	2	09/01/83	03/21/85
2119	1	09/01/82	03/21/85
2124	1	01/01/70	03/22/85
2125	2	01/01/65	03/22/85
2126	2*	01/01/75	03/22/85
2129	2	01/01/76	03/22/85

Number	Sex	Date of Birth	Date of Entry
2134	2	05/01/82	03/28/85
2135	2	06/01/83	03/28/85
2138	1	01/01/70	03/28/85
2141	2	01/01/67	04/02/85
2142	2	01/01/80	04/02/85
2143	1	01/01/76	04/02/85
2144	2	01/01/65	05/21/85
2145	2	01/01/65	05/21/85
2147	2	01/01/79	04/04/85
2149	2	01/01/65	04/04/85
2150	2	01/01/65	04/04/85
2151	2	01/01/72	04/10/85
2152	2	01/01/64	04/10/85
2153	1	01/01/64	04/10/85
2155	2	01/01/45	04/10/85
2156	1*	01/01/72	04/10/85
2157	2	01/01/72	04/10/85
2158	1	01/01/72	04/10/85
2161	1	01/01/80	04/17/85
2164	2	01/01/76	04/17/85
2166	1	01/01/70	04/24/85
2167	1	01/01/75	04/24/85
2168	2	01/01/71	04/24/85
2169	1	01/01/75	04/24/85
2170	1	01/01/69	04/24/85
2171	1	01/01/73	04/24/85
2172	2	01/01/65	04/24/85
2173	1	01/01/71	04/24/85
2174	2*	01/01/65	04/24/85
2176	1	01/01/69	04/25/85
2177	2	01/01/79	04/25/85
2178	2	01/01/77	04/25/85
2179	2	01/01/71	04/25/85
2180	2	01/01/73	04/25/85
2181	2	01/01/80	04/25/85
2182	2*	01/01/71	04/25/85
2183	1	01/01/83	04/25/85
2185	2	01/01/69	04/30/85
2186	2	01/01/74	04/30/85
2187	2*	01/01/73	04/30/85
2188	1	01/01/69	05/02/85
2190	2	01/01/65	05/02/85
2195	1	01/01/75	05/03/85
2198	1*	01/01/61	05/07/85
2201	2	01/01/81	05/10/85
2203	1	01/01/67	05/10/85
2205	2*	01/01/70	05/10/85
2206	2	01/01/60	05/10/85
2210	1	01/01/75	05/14/85
2211	2	01/01/73	05/14/85
2215	2	05/01/85	05/10/85
2221	1	01/01/79	05/17/85

Number	Sex	Date of Birth	Date of Entry
2228	1	01/01/75	05/23/85
2229	2	01/01/80	05/23/85
2232	2*	01/01/73	05/29/85
2234	2	01/01/55	05/29/85
2235	1	01/01/75	05/29/85
2236	2	01/01/70	05/29/85
2237	1	01/01/60	05/29/85
2244	1	01/01/70	06/12/85
2247	1	01/01/73	06/14/85
2248	2	01/01/78	06/14/85
2249	1	01/01/72	06/14/85
2251	2	01/01/74	06/21/85
2252	2*	01/01/76	06/21/85
2253	2	01/01/78	06/21/85
2251	2	01/01/74	06/21/85
2252	2*	01/01/76	06/21/85
2253	2	01/01/78	06/21/85
2254	1	01/01/72	06/26/85
2258	1	01/01/71	06/26/85
2259	1	01/01/74	06/26/85
2265	2	01/01/69	06/28/85
2266	1	01/01/73	07/05/85
2267	1	01/01/69	07/05/85
2268	1	01/01/79	07/05/85
2277	1	01/01/77	07/10/85
2278	2	01/01/79	07/10/85
2280	1	01/01/80	07/12/85
2281	1	01/01/80	07/12/85
2283	2	01/01/74	07/17/85
2284	2*	01/01/67	07/17/85
2285	2	01/01/69	07/17/85
2287	1	01/01/70	07/18/85
2288	1	07/01/75	07/18/85
2290	1	01/01/80	07/23/85
2294	1	01/01/60	07/23/85
2295	2	01/01/77	07/25/85
2296	1	01/01/65	07/25/85
2297	1	01/01/57	07/29/85
2298	1	01/01/71	07/29/85
2299	1	01/01/71	07/29/85
2300	1	01/01/65	08/02/85
2301	1	01/01/80	08/02/85
2302	2	01/01/73	08/02/85
2303	1*	01/01/67	08/02/85
2306	2*	01/01/65	08/14/85
2307	1*	01/01/70	08/14/85
2311	2*	01/01/69	08/30/85
2314	1	01/01/65	08/30/85
2315	1*	01/01/64	07/08/85
2317	2	01/01/72	09/03/85
2318	2	01/01/74	09/03/85
2324	2	01/01/68	09/06/85

Number	Sex	Date of Birth	Date of Entry
2328	1	01/01/65	09/11/85
2329	1	01/01/72	09/11/85
2330	1*	01/01/82	09/11/85
2333	1	01/01/60	09/11/85
2335	1	01/01/62	09/13/85
2336	2*	09/01/65	09/13/85
2337	2*	09/01/60	09/13/85
2339	1	01/01/65	09/13/85
2341	2	01/01/77	09/19/85
2342	2*	01/01/65	09/19/85
2343	2	01/01/68	09/19/85
2347	1	01/01/65	09/25/85
2348	2	01/01/55	09/25/85
2351	1	01/01/58	09/27/85
2352	1*	01/01/45	09/27/85
2354	1*	01/01/55	09/27/85
2355	1	01/01/80	09/27/85
2356	1	01/01/63	10/04/85
2357	1	01/01/67	10/04/85
2358	2*	01/01/74	11/15/85
2362	2*	01/01/55	11/20/85
2363	2	01/01/72	11/20/85
2368	2*	01/01/50	10/09/85
2370	2	10/01/60	10/16/85
2373	1	01/01/69	10/16/85
2376	1	01/01/57	10/16/85
2383	1	01/01/67	10/22/85
2384	1	01/01/67	10/22/85
2386	2*	01/01/74	10/22/85
2391	2	01/01/66	10/22/85
2392	2*	01/01/77	10/22/85
2393	1	01/01/79	10/22/85
2394	2	01/01/72	10/22/85
2395	2	01/01/79	10/22/85
2396	1	01/01/81	10/22/85
2397	2	01/01/50	10/22/85
2398	1	01/01/76	10/24/85
2399	1	01/01/74	10/24/85
2401	2	01/01/75	10/24/85
2402	1	01/01/65	10/24/85
2403	2	01/01/65	10/24/85
2404	2	01/01/58	10/24/85
2405	1	01/01/65	10/24/85
2410	1	01/01/79	10/31/85
2411	2	01/01/60	10/31/85
2412	1	01/01/60	10/31/85
2413	2	01/01/66	10/31/85
2414	1	01/01/65	10/31/85
2416	2	01/01/72	11/06/85
2417	2	01/01/60	11/06/85
2418	2	01/01/72	11/06/85
2420	2	01/01/77	11/06/85

Number	Sex	Date of Birth	Date of Entry
2433	2	01/01/68	11/12/85
2434	1	01/01/60	11/12/85
2436	2	05/01/82	11/14/85
2438	1	07/01/85	11/14/85
2440	2	01/01/70	11/14/85
2442	2*	01/01/73	11/14/85
2443	1	09/01/85	11/14/85
2444	2*	01/01/73	11/15/85
2445	2*	01/01/62	11/15/85
2449	1	01/01/55	11/18/85
2450	1	01/01/65	11/18/85
2451	1	01/01/72	11/20/85
2452	2	01/01/55	11/22/85
2453	2*	01/01/60	11/22/85
2455	2	01/01/65	11/20/85
2459	1	01/01/80	04/17/85
2462	2	01/01/70	10/04/85
2467	1*	01/01/69	11/08/85
2468	1	01/01/72	11/29/85
2469	1	01/01/72	11/29/85
2478	1	01/01/65	12/10/85
2480	1	01/01/78	12/10/85
2481	2	01/01/67	12/10/85
2483	2*	01/01/60	12/12/85
2487	1	01/01/66	11/29/85
2488	1	01/01/66	11/29/85
2489	1	01/01/76	12/13/85
2490	2	01/01/70	12/13/85
2491	2	01/01/82	12/19/85
2492	1	01/01/60	12/19/85
2503	2	01/01/69	01/07/86
2504	1	01/01/80	01/07/86
2505	1	01/01/71	01/07/86
2508	1	01/01/73	01/08/86
2509	1	01/01/81	01/08/86
2516	2	01/01/78	01/13/86
2517	2	01/01/74	01/13/86
2518	2	01/01/72	01/13/86
2525	2	01/01/68	01/21/86
2526	1	01/01/76	01/21/86
2530	2	01/01/77	01/23/86
2531	1	01/01/76	01/20/86
2532	2*	01/01/46	01/30/86
2533	1	01/01/69	01/31/86
2534	2	01/01/84	01/31/86
2536	2	01/01/72	01/31/86
2537	2	01/01/81	01/31/86
2538	1	01/01/73	01/10/86
2547	2*	01/01/66	02/07/86
2548	2	01/01/51	02/07/86
2551	1	01/01/78	02/12/86
2552	2	01/01/74	02/12/86

Number	Sex	Date of Birth	Date of Entry
2556	2*	01/01/68	02/13/86
2557	2	01/01/68	02/13/86
2558	1	01/01/70	02/19/86
2559	2	01/01/71	02/19/86
2563	2	01/01/74	02/21/86
2564	2	01/01/76	02/21/86
2565	2*	01/01/58	02/21/86
2566	1	01/01/78	02/26/86
2567	2	06/01/84	02/26/86
2568	1	01/01/80	02/26/86
2571	1	01/01/68	03/27/86
2573	2	01/01/67	02/04/86
2575	1	01/01/60	03/05/86
2576	2	01/01/56	03/12/86
2577	2	01/01/78	03/12/86
2578	1	06/01/81	03/12/86
2579	1	01/01/76	03/12/86
2580	1	01/01/75	03/12/86
2585	1	01/01/75	03/21/86
2586	2	01/01/72	03/21/86
2588	1	01/01/62	03/21/86
2593	1*	01/01/58	03/21/86
2594	2	01/01/76	03/24/86
2595	1	01/01/73	03/24/86
2596	1	01/01/70	03/24/86
2597	1	01/01/72	03/25/86
2600	1	01/01/80	03/25/86
2601	2	01/01/70	03/25/86
2602	1	01/01/56	03/24/86
2603	2	08/01/83	03/24/86
2604	1	01/01/66	03/27/86
2605	2	01/01/73	03/27/86
2606	2	01/01/56	03/27/86
2608	2	01/01/78	04/02/86
2609	1	03/01/85	04/02/86
2610	1	04/01/85	04/02/86
2611	2	01/01/70	04/02/86
2612	2	01/01/60	04/02/86
2613	2	01/01/71	04/02/86
2614	1	01/01/61	04/02/86
2615	2	01/01/78	04/02/86
2616	1	01/01/56	04/02/86
2617	1	01/01/77	04/02/86
2618	1	01/01/56	04/02/86
2619	1	01/01/56	04/02/86
2620	2	01/01/82	04/02/86
2621	2	01/01/56	04/02/86
2622	1	01/01/77	03/13/86
2623	1	01/01/78	03/13/86
2624	2	01/01/60	04/09/86
2628	1	05/01/69	04/08/86
2629	2	05/01/69	04/08/86

Number	Sex	Date of Birth	Date of Entry
2633	1	01/01/48	04/20/86
2636	1*	01/01/69	04/20/86
2639	1	01/01/73	04/16/86
2640	1*	01/01/60	04/16/86
2643	1	01/01/74	04/23/86
2644	2	01/01/68	04/23/86
2645	2	01/01/83	04/23/86
2646	2	01/01/80	04/23/86
2647	1	10/01/85	04/23/86
2650	2	01/01/66	04/24/86
2651	1	01/01/75	04/24/86
2655	1	04/01/86	04/21/86
2656	2	01/01/66	04/25/86
2661	1	01/01/60	04/29/86
2662	1	01/01/65	04/29/86
2663	1	01/01/81	05/13/86
2668	2	01/01/83	05/16/86
2669	2	01/01/72	05/16/86
2670	1	01/01/61	05/16/86
2671	2*	01/01/46	05/16/86
2672	2*	01/01/66	05/16/86
2674	2	01/01/84	05/19/86
2675	2	01/01/61	05/19/86
2676	1	01/01/80	05/19/86
2677	2	01/01/69	05/16/86
2679	2	07/01/85	05/22/86
2680	2	04/01/83	05/22/86
2682	1	01/01/61	05/22/86
2686	1	01/01/70	05/19/86
2691	2	01/01/56	06/03/86
2694	1	05/01/86	05/31/86
2695	2*	01/01/71	06/12/86
2698	2	01/01/71	06/12/86
2699	1	01/01/59	06/13/86
2702	1	01/01/78	06/18/86
2703	2	01/01/82	06/18/86
2704	1	01/01/61	06/18/86
2708	1	01/01/66	06/25/86
2713	2	05/01/86	06/25/86
2714	1	01/01/79	06/30/86
2721	2*	01/01/46	07/08/86
2722	1*	01/01/46	07/08/86
2726	2	01/01/74	07/11/86
2727	2	01/01/81	07/11/86
2728	1	01/01/76	07/11/86
2731	2*	01/01/60	07/15/86
2733	2	01/01/81	07/18/86
2736	1	01/01/85	07/18/86
2737	2	01/01/84	07/18/86
2738	2	01/01/58	06/06/86
2739	2	01/01/53	06/06/86
2744	2	01/01/74	07/22/86

Number	Sex	Date of Birth	Date of Entry
2750	1	01/01/78	07/29/86
2751	2	01/01/77	07/31/86
2755	1	01/01/68	08/08/86
2756	2	01/01/68	08/08/86
2757	2	01/01/73	08/08/86
2758	2	01/01/73	08/08/86
2761	2*	01/01/49	08/12/86
2763	2	01/01/73	08/13/86
2764	1	01/01/84	08/13/86
2765	1	01/01/82	08/13/86
2768	2	01/01/60	08/18/86
2769	1	01/01/77	08/18/86
2770	1	01/01/60	08/18/86
2772	1	01/01/56	08/22/86
2773	1	01/01/61	08/22/86
2774	1	01/01/67	08/22/86
2775	2	01/01/77	08/22/86
2776	1	01/01/68	08/22/86
2777	1	01/01/77	08/14/86
2778	1	01/01/77	08/14/86
2779	1	01/01/62	08/21/86
2780	2	01/01/65	08/21/86
2782	2	01/01/71	08/27/86
2783	1	01/01/67	08/27/86
2785	1	01/01/75	09/02/86
2786	2*	01/01/61	09/05/86
2787	2	01/01/68	09/05/86
2788	2	01/01/76	09/05/86
2789	1	01/01/73	09/05/86
2790	2	01/01/82	09/05/86
2792	2	01/01/61	09/11/86
2793	2	01/01/61	09/11/86
2794	2	01/01/70	09/11/86
2798	1	01/01/76	09/19/86
2803	1	01/01/61	09/23/86
2804	1	01/01/86	09/23/86
2805	1	01/01/61	09/23/86
2815	2	01/01/56	10/01/86
2816	1	01/01/66	10/01/86
2821	1	05/01/80	09/04/86
2822	2	01/01/82	09/04/86
2824	1	01/01/67	10/09/86
2825	1*	01/01/62	10/09/86
2827	2	01/01/78	09/04/86
2828	1	01/01/74	10/07/86
2829	1	01/01/75	10/07/86
2830	1	01/01/76	10/07/86
2831	1	01/01/82	10/07/86
2834	1	01/01/74	10/09/86
2836	1	01/01/66	10/09/86
2838	2*	10/01/66	10/17/86
2841	1*	01/01/82	10/01/86

Number	Sex	Date of Birth	Date of Entry
2854	1	01/01/79	09/24/86
2856	2	07/01/76	11/04/86
2857	2	04/01/72	11/04/86
2860	2	01/01/78	11/04/86
2861	2	01/01/78	11/04/86
2862	1	01/01/73	11/04/86
2867	2	01/01/51	11/06/86
2868	1*	01/01/51	11/06/86
2869	1	01/01/51	11/11/86
2871	1*	01/01/61	11/14/86
2875	1	01/01/74	11/21/86
2882	1	01/01/61	11/26/86
2884	1*	01/01/61	11/26/86
2885	2	01/01/68	11/26/86
2886	1	01/01/75	11/26/86
2887	2	06/01/85	11/26/86
2888	1*	01/01/70	12/02/86
2889	2	01/01/79	12/04/86
2892	2*	01/01/71	12/05/86
2893	2*	01/01/56	12/05/86
2896	1	01/01/70	12/02/86
2900	2*	01/01/61	12/10/86
2901	2	01/01/71	12/16/86
2902	2	01/01/66	12/16/86
2903	2	01/01/79	12/16/86
2904	1	01/01/69	12/16/86
2908	2*	01/01/61	12/11/86
2909	1	01/01/68	12/16/86
2910	1	01/01/82	12/16/86
2911	2	01/01/74	12/16/86
2912	2	01/01/68	12/16/86
2914	1	01/01/51	12/19/86
2915	1*	01/01/59	12/19/86
2916	2*	01/01/61	12/19/86
2919	1	01/01/82	12/19/86
2920	2	01/01/82	12/19/86
2921	1*	01/01/61	12/19/86
2922	1*	01/01/61	12/23/86
2923	1*	01/01/59	12/23/86
2924	2*	01/01/47	12/23/86
2925	1*	01/01/51	12/23/86
2927	2	01/01/60	12/31/86
2933	2	01/01/85	01/21/87
2934	2	01/01/82	01/21/87
2935	2	01/01/84	01/21/87
2936	2	01/01/64	01/21/87
2937	1	01/01/63	01/21/87
2938	2*	01/01/60	01/23/87
2941	1	01/01/68	01/23/87
2942	1	01/01/61	01/28/87
2943	1	01/01/71	01/28/87
2944	1*	01/01/52	01/28/87

Number	Sex	Date of Birth	Date of Entry
2951	1	01/01/69	02/03/87
2952	1	01/01/75	02/03/87
2954	1	05/01/86	02/06/87
2955	2	01/01/64	02/06/87
2960	1	01/01/61	02/13/87
2961	1	01/01/81	02/17/87
2962	2*	01/01/47	02/17/87
2963	2*	01/01/47	02/17/87
2964	2	06/01/69	02/20/87
2965	1	01/01/63	02/20/87
2966	2	01/01/78	02/27/87
2967	1	03/01/85	02/27/87
2970	1*	01/01/52	02/27/87
2971	1	01/01/59	02/27/87
2973	2	01/01/50	02/27/87
2974	2	01/01/68	02/27/87
2976	1	01/01/62	03/03/87
2977	1	01/01/75	03/03/87
2978	1	01/01/62	03/11/87
2979	1	01/01/66	03/12/87
2980	1	01/01/71	12/10/86
2984	1	01/01/58	03/17/87
2988	1	04/01/74	03/20/87
2989	1	07/01/86	03/17/87
2991	2	01/01/57	03/27/87
2992	2	01/01/62	03/27/87
2993	2	01/01/50	03/27/87
2995	2	01/01/73	04/02/87
2996	2	01/01/83	04/02/87
2997	1	01/01/77	03/24/87
3000	1	04/01/87	04/05/87
3001	2	01/01/58	04/08/87
3003	2	01/01/77	04/08/87
3004	2	01/01/83	04/08/87
3005	1	01/01/70	04/14/87
3009	2	01/01/69	04/14/87
3010	1	01/01/76	04/15/87
3016	1	01/01/70	04/28/87
3019	1	01/01/73	04/21/87
3020	2	01/01/73	05/12/87
3021	2	01/01/65	05/12/87
3022	2	01/01/62	05/12/87
3023	2	01/01/81	05/12/87
3024	1	01/01/75	04/24/87
3026	2	01/01/66	04/21/87
3027	2	01/01/77	04/21/87
3028	1	01/01/86	04/21/87
3034	2	01/01/84	05/12/87
3035	1	01/01/81	05/06/87
3036	1	01/01/54	05/06/87
3038	2	01/01/57	05/07/87
3039	2	01/01/72	05/07/87

Number	Sex	Date of Birth	Date of Entry
3047	1	01/01/75	05/05/87
3049	2	05/19/87	05/19/87
3050	2	01/01/81	05/22/87
3052	1	01/01/52	05/27/87
3053	2	01/01/52	05/27/87
3054	1	01/01/77	05/27/87
3055	1	01/01/64	05/20/87
3057	2	01/01/71	05/29/87
3058	1	01/01/71	05/29/87
3060	2	01/01/83	05/29/87
3061	2	01/01/72	05/29/87
3063	2	01/01/52	05/29/87
3064	2*	01/01/50	05/29/87
3065	1	01/01/62	06/05/87
3066	1	04/01/85	06/05/87
3071	2*	01/01/76	06/05/87
3072	2	01/01/67	06/05/87
3076	1	01/01/62	06/12/87
3077	1	01/01/58	06/12/87
3080	2*	01/01/67	06/12/87
3081	2	01/01/81	06/12/87
3082	2	01/01/52	06/19/87
3083	2*	01/01/52	06/19/87
3084	2	01/01/79	06/18/87
3085	1*	01/01/67	06/18/87
3086	2	01/01/62	06/18/87
3087	2	01/01/65	06/18/87
3088	2	01/01/70	06/24/87
3089	2	01/01/62	06/24/87
3090	2	01/01/85	06/24/87
3091	2*	01/01/67	06/24/87
3092	1	01/01/74	06/24/87
3093	2*	01/01/51	06/24/87
3097	2*	01/01/62	06/26/87
3098	1	09/01/67	06/29/87
3099	2	01/01/70	07/02/87
3100	1	01/01/60	07/02/87
3102	2	01/01/57	06/29/87
3107	2*	01/01/80	07/03/87
3108	2	06/01/87	07/03/87
3109	2	01/01/85	07/03/87
3111	2*	01/01/70	07/03/87
3113	2*	01/01/85	07/03/87
3115	1	01/01/60	07/06/87
3116	1	01/01/65	07/09/87
3120	1	01/01/52	07/14/87
3121	1	01/01/55	07/14/87
3122	1	01/01/82	07/14/87
3123	1	01/01/85	07/14/87
3124	1	01/01/84	07/14/87
3129	2	01/01/72	07/17/87
3130	2	01/01/81	07/21/87

Number	Sex	Date of Birth	Date of Entry
3134	1	01/01/80	07/23/87
3135	2	01/01/83	07/23/87
3136	1	01/01/81	07/23/87
3137	2	01/01/60	07/23/87
3138	1	01/01/84	07/24/87
3139	1	01/01/72	07/24/87
3140	1	01/01/69	07/28/87
3141	1	01/01/65	07/28/87
3143	2	01/01/52	07/24/87
3144	2	01/01/62	07/24/87
3145	2	07/01/87	07/25/87
3147	1	01/01/75	07/28/87
3148	2	01/01/84	07/22/87
3149	2	01/01/83	07/22/87
3150	2	01/01/72	07/22/87
3152	2	01/01/83	07/22/87
3153	2	01/01/83	07/22/87
3154	2	01/01/80	07/22/87
3155	2	01/01/75	07/22/87
3156	2	01/01/82	07/22/87
3157	1*	01/01/60	07/24/87
3158	1	01/01/69	07/24/87
3159	1	01/01/73	07/29/87
3162	1	10/01/68	08/07/87
3163	1	01/01/72	08/07/87
3164	2	01/01/72	08/12/87
3166	2	01/01/52	08/15/87
3167	1	01/01/76	08/21/87
3168	2	01/01/71	08/26/87
3169	1	01/01/72	08/26/87
3170	2	09/01/82	08/26/87
3166	2	01/01/52	08/15/87
3167	1	01/01/76	08/21/87
3168	2	01/01/71	08/26/87
3169	1	01/01/72	08/26/87
3170	2	09/01/82	08/26/87
3172	1	01/01/57	09/02/87
3185	2	01/01/73	09/10/87
3186	1	01/01/75	09/10/87
3187	2	01/01/69	09/10/87
3188	1	01/01/78	09/11/87
3191	2	01/01/72	09/11/87
3192	2	01/01/72	09/11/87
3193	2	01/01/76	09/16/87
3194	1	01/01/82	09/18/87
3195	1	01/01/75	09/18/87
3196	2	01/01/75	09/18/87
3197	1	01/01/62	09/18/87
3198	2	01/01/72	09/18/87
3199	1	01/01/57	09/18/87
3200	2	01/01/79	09/18/87
3201	1	01/01/87	09/18/87

Number	Sex	Date of Birth	Date of Entry
3206	2	01/01/78	09/01/87
3207	2	01/01/78	09/25/87
3208	2	01/01/77	09/25/87
3211	2	01/01/61	09/29/87
3217	2	01/01/74	10/06/87
3218	2	01/01/72	10/06/87
3220	2	01/01/77	10/06/87
3221	2	01/01/62	10/06/87
3223	1	01/01/57	10/13/87
3228	2	01/01/59	10/16/87
3229	1	01/01/72	10/16/87
3232	2	01/01/83	10/16/87
3234	1	10/01/87	10/19/87
3238	1	01/01/67	10/28/87
3239	2	01/01/70	10/28/87
3240	2	01/01/76	10/28/87
3241	2*	01/01/57	10/28/87
3243	1	01/01/80	10/29/87
3244	2	01/01/62	10/31/87
3245	1	01/01/74	10/31/87
3246	1	01/01/75	10/31/87
3247	2	01/01/67	10/31/87
3248	1	01/01/62	10/31/87
3250	1	01/01/83	09/14/87
3252	1	01/01/62	11/03/87
3253	1	01/01/60	11/06/87
3254	2	01/01/74	11/13/87
3255	2	01/01/83	11/13/87
3257	1*	01/01/65	11/13/87
3258	2	01/01/62	11/13/87
3264	2	01/01/79	10/27/87
3265	1	01/01/62	11/18/87
3268	2	01/01/80	11/07/87
3269	1*	01/01/77	11/17/87
3270	2*	01/01/62	11/17/87
3272	2	01/01/57	11/17/87
3278	2*	01/01/47	11/20/87
3279	1	01/01/66	11/20/87
3283	2	01/01/57	11/25/87
3284	1	01/01/72	11/25/87
3285	1*	01/01/52	11/25/87
3286	2	01/01/63	12/01/87
3287	1	01/01/68	12/01/87
3288	1	01/01/61	12/01/87
3289	1	01/01/73	12/01/87
3294	1	01/01/84	12/04/87
3295	1	01/01/85	12/04/87
3296	1	01/01/82	12/04/87
3298	1	01/01/47	12/08/87
3299	1	01/01/49	12/08/87
3301	2	01/01/57	12/11/87
3309	2	01/01/80	12/13/87

Number	Sex	Date of Birth	Date of Entry
3323	2	07/01/74	12/30/87
3324	2	01/01/58	12/29/87
3325	1	01/01/58	12/29/87
3326	1	07/01/69	01/06/88
3327	2	01/01/76	01/06/88
3330	2	01/01/74	01/06/88
3331	2	01/01/63	01/03/88
3332	1	01/01/77	01/09/88
3333	1	01/01/72	01/12/88
3334	2	01/01/60	01/12/88
3335	1	01/01/59	01/14/88
3336	2	01/01/63	01/19/88
3338	2	01/01/68	01/22/88
3340	1	01/01/60	01/22/88
3341	2	01/01/58	01/22/88
3342	1	01/01/76	01/22/88
3343	2	01/01/61	01/26/88
3344	2	01/01/68	01/26/88
3347	2	01/01/60	01/29/88
3350	1	01/01/84	01/29/88
3351	2	01/01/83	02/02/88
3352	1	06/01/87	02/02/88
3353	2	01/01/84	02/02/88
3354	2	06/01/87	02/02/88
3355	2	01/01/63	02/05/88
3356	1	01/01/70	02/05/88
3357	1	01/01/47	02/05/88
3358	1	01/01/78	02/05/88
3359	1	01/01/69	02/05/88
3360	1	01/01/78	02/02/88
3362	1	01/01/53	02/05/88
3363	2	01/01/61	02/09/88
3364	1	01/01/87	02/09/88
3366	2	01/01/53	02/12/88
3367	1	01/01/69	02/09/88
3368	1	01/01/67	02/09/88
3369	2*	01/01/53	02/15/88
3370	1	01/01/73	02/17/88
3372	1	01/01/63	02/25/88
3373	1*	01/01/48	02/25/88
3374	2	01/01/53	02/25/88
3375	2	01/01/55	02/25/88
3380	2	01/01/68	03/02/88
3381	1	01/01/57	03/02/88
3382	1	01/01/78	03/01/88
3383	1	01/01/63	03/04/88
3385	1	01/01/60	03/04/88
3386	1	01/01/68	03/04/88
3388	2	01/01/58	03/09/88
3389	2	01/01/68	03/09/88
3390	2	01/01/77	03/09/88
3391	1	01/01/78	03/10/88

Number	Sex	Date of Birth	Date of Entry
3397	1	01/01/63	03/18/88
3398	1	01/01/63	03/18/88
3403	2	01/01/71	03/23/88
3404	2	01/01/63	03/23/88
3405	2	01/01/60	03/23/88
3410	2	01/01/63	03/25/88
3411	2	01/01/58	03/25/88
3412	2	01/01/53	03/25/88
3413	1	01/01/63	03/25/88
3414	2	01/01/58	03/25/88
3415	1	01/01/84	03/25/88
3416	1	01/01/80	03/24/88
3417	1	01/01/84	03/24/88
3418	1	01/01/61	03/30/88
3420	1	01/01/87	04/08/88
3421	2	01/01/68	04/08/88
3422	1	01/01/63	04/12/88
3425	1	01/01/74	04/12/88
3427	2	01/01/60	04/15/88
3430	2	01/01/78	04/15/88
3434	1	01/01/82	04/15/88
3435	1	01/01/84	04/15/88
3436	1	01/01/77	04/15/88
3437	1	01/01/70	04/20/88
3439	1	01/01/68	04/26/88
3440	1	01/01/63	04/26/88
3441	1	01/01/58	04/26/88
3443	2	01/01/73	04/29/88
3445	1	01/01/80	05/03/88
3448	1	01/01/70	04/18/88
3449	1	01/01/72	05/13/88
3450	1	01/01/63	05/09/88
3451	1	01/01/71	05/18/88
3452	1	01/01/63	05/18/88
3453	2	01/01/62	05/18/88
3454	1*	01/01/48	05/18/88
3455	1	01/01/50	05/18/88
3456	1	01/01/83	05/18/88
3457	2	01/01/76	05/18/88
3460	1	01/01/46	05/19/88
3461	1	01/01/85	05/18/88
3462	1	01/01/62	05/18/88
3463	2	01/01/87	05/18/88
3464	2	01/01/53	05/18/88
3465	2	01/01/61	05/25/88
3466	2	01/01/58	05/25/88
3467	2	01/01/63	05/25/88
3469	2	01/01/58	05/25/88
3471	2	01/01/60	05/25/88
3472	2	01/01/64	05/25/88
3473	2	01/01/63	05/20/88
3474	2	01/01/63	05/20/88

Number	Sex	Date of Birth	Date of Entry
3479	1	01/01/72	06/02/88
3480	2*	01/01/56	06/03/88
3481	1	10/01/60	06/07/88
3483	1	01/01/85	06/10/88
3484	2	01/01/83	06/10/88
3485	1	01/01/83	06/24/88
3486	1	01/01/80	06/24/88
3487	2	01/01/75	06/24/88
3488	1	01/01/79	06/24/88
3490	2	06/01/88	06/08/88
3491	2	06/01/88	06/09/88
3492	1*	01/01/58	06/15/88
3493	2	01/01/58	06/15/88
3494	2	01/01/73	06/15/88
3495	1	01/01/58	06/15/88
3497	1	01/01/75	06/21/88
3498	2	01/01/70	06/21/88
3500	2	01/01/63	06/24/88
3501	2	01/01/68	06/24/88
3502	1	01/01/83	06/24/88
3503	2	01/01/63	06/22/88
3504	1	01/01/76	06/22/88
3505	1*	01/01/76	06/22/88
3506	2	01/01/63	07/01/88
3508	2	01/01/60	06/29/88
3510	2	01/01/71	07/01/88
3511	1	01/01/78	07/05/88
3512	1	01/01/83	07/05/88
3518	1	01/01/58	07/07/88
3519	2*	01/01/76	07/07/88
3520	2	01/01/82	07/07/88
3523	2	01/01/78	07/14/88
3527	2	01/01/68	07/22/88
3528	1	01/01/70	07/22/88
3529	2	01/01/63	09/23/88
3530	1	01/01/76	09/23/88
3531	2	01/01/58	07/22/88
3532	2	01/01/69	07/21/88
3533	2	01/01/86	07/22/88
3534	2	01/01/63	07/21/88
3535	1	01/01/74	07/21/88
3536	2	01/01/76	07/21/88
3537	1	01/01/70	07/21/88
3539	1	01/01/68	07/21/88
3540	1	01/01/58	07/28/88
3541	1	01/01/63	07/28/88
3542	2*	01/01/63	07/28/88
3543	1	01/01/70	07/28/88
3544	2	01/01/84	07/28/88
3545	2	01/01/87	07/28/88
3546	2	01/01/73	07/28/88
3547	2	01/01/86	07/28/88

Number	Sex	Date of Birth	Date of Entry
3559	2	01/01/74	08/05/88
3566	1	01/01/73	08/09/88
3568	1	01/01/53	08/12/88
3569	2	01/01/76	08/12/88
3572	1	01/01/70	08/17/88
3575	1	01/01/75	08/19/88
3576	1	01/01/58	08/19/88
3577	2	01/01/64	08/19/88
3578	2	01/01/70	08/19/88
3580	2	01/01/80	08/08/88
3581	1*	01/01/61	08/19/88
3582	1	01/01/74	08/25/88
3583	1	01/01/63	08/25/88
3584	1	01/01/70	08/25/88
3586	1	01/01/76	08/25/88
3592	1	01/01/80	09/02/88
3593	2	01/01/48	09/02/88
3594	2	01/01/68	09/02/88
3597	1	01/01/76	09/07/88
3599	2	01/01/76	09/09/88
3600	2	01/01/74	09/09/88
3601	2	01/01/48	09/13/88
3605	2	01/01/78	09/13/88
3606	1	01/01/66	09/13/88
3607	1	01/01/58	09/09/88
3609	1	01/01/68	09/13/88
3610	2	01/01/79	09/15/88
3611	2	01/01/79	09/15/88
3612	1	01/01/69	09/15/88
3613	2	01/01/73	09/15/88
3614	1	01/01/73	09/15/88
3615	2	01/01/74	09/15/88
3620	2	01/01/75	09/22/88
3621	1	01/01/78	09/23/88
3622	2	01/01/63	09/27/88
3623	1	01/01/67	09/27/88
3624	2*	01/01/48	09/27/88
3625	1	01/01/63	09/29/88
3627	1	01/01/85	09/29/88
3630	2	01/01/82	10/04/88
3631	2	01/01/48	10/04/88
3632	1	01/01/73	10/04/88
3633	2	01/01/63	10/06/88
3634	1*	01/01/63	10/07/88
3635	1	01/01/68	10/07/88
3636	1	01/01/60	10/07/88
3637	1	01/01/58	10/07/88
3638	2*	01/01/80	10/07/88
3639	2*	01/01/48	10/07/88
3643	2	01/01/61	10/11/88
3644	1	01/01/78	10/11/88
3645	2*	01/01/48	10/20/88

Number	Sex	Date of Birth	Date of Entry
3649	1*	01/01/82	10/20/88
3650	2	01/01/64	10/20/88
3651	1	08/01/88	10/20/88
3652	2	01/01/84	10/20/88
3653	1	04/01/88	10/20/88
3654	2	01/01/79	10/20/88
3655	2	09/01/88	10/20/88
3656	2	01/01/77	10/20/88
3657	1	08/01/88	10/20/88
3658	1	01/01/79	10/19/88
3659	2	01/01/81	11/04/88
3662	2	01/01/82	11/09/88
3663	2*	01/01/63	11/09/88
3664	2	01/01/79	11/09/88
3665	1	01/01/77	11/09/88
3666	2	01/01/63	11/09/88
3670	2	01/01/78	11/11/88
3671	2	01/01/65	11/09/88
3672	2	01/01/73	11/09/88
3673	1	01/01/63	11/09/88
3674	1	09/01/79	11/09/88
3676	2	01/01/73	11/15/88
3677	1	01/01/68	11/09/88
3678	1	01/01/53	11/16/88
3679	2	01/01/68	11/16/88
3680	2	01/01/72	11/16/88
3681	1	01/01/48	11/16/88
3682	2	01/01/68	11/15/88
3683	1	01/01/63	11/15/88
3684	2	01/01/58	11/15/88
3685	1	01/01/53	11/18/88
3686	1	01/01/52	11/22/88
3687	1	01/01/74	11/23/88
3688	2	01/01/58	11/23/88
3689	1*	01/01/76	11/23/88
3690	1*	01/01/62	11/28/88
3691	2	01/01/81	11/24/88
3692	1	01/01/76	11/24/88
3693	2*	01/01/48	12/01/88
3694	2	01/01/84	12/01/88
3695	2	01/01/78	12/01/88
3697	2	01/01/85	12/01/88
3698	2	01/01/87	12/01/88
3699	2	01/01/76	12/01/88
3701	2	01/01/79	12/01/88
3702	2	01/01/87	12/01/88
3703	2	01/01/76	12/01/88
3704	2	01/01/87	12/01/88
3705	2	01/01/48	12/01/88
3706	2	01/01/64	12/01/88
3708	1	01/01/63	12/01/88
3709	2	09/01/71	12/06/88

Number	Sex	Date of Birth	Date of Entry
3715	2	01/01/74	12/06/88
3716	1	01/01/69	12/03/88
3720	2	01/01/54	12/13/88
3721	2	01/01/69	12/13/88
3722	2	01/01/70	12/13/88
3723	2	01/01/54	12/13/88
3724	2	01/01/53	12/20/88
3725	2	01/01/69	12/19/88
3726	2	01/01/68	12/19/88
3728	1	01/01/84	12/13/88
3731	1	01/01/79	12/14/88
3732	1	01/01/74	12/19/88
3733	1	01/01/59	12/19/88
3734	2	01/01/70	12/19/88
3735	2	01/01/71	12/19/88
3736	1	01/01/82	12/14/88
3738	1	01/01/73	12/12/88
3740	2	01/01/81	01/05/89
3741	1	07/01/88	01/05/89
3742	2	01/01/87	01/05/89
3743	2	01/01/49	01/05/89
3744	2	01/01/85	01/05/89
3750	1	01/01/67	01/31/89
3751	2	01/01/49	01/31/89
3752	2	01/01/62	01/12/89
3754	2	12/01/88	12/25/88
3755	2	01/01/51	01/10/89
3756	1	10/01/88	01/10/89
3757	1	01/01/84	01/16/89
3758	1	01/01/79	01/12/89
3759	2	01/01/65	01/12/89
3761	2	01/01/54	01/12/89
3762	1	01/01/69	01/16/89
3763	1	01/01/69	01/16/89
3764	2	01/01/84	01/16/89
3765	1	01/01/88	01/16/89
3766	2	01/01/75	01/16/89
3767	2	01/01/54	01/20/89
3768	1	01/01/81	01/24/89
3769	2	01/14/89	01/14/89
3770	1	01/01/88	01/23/89
3773	2	01/01/85	01/23/89
3774	2	01/01/75	01/23/89
3776	2	01/01/86	02/01/89
3778	2	01/01/88	03/30/89
3779	1	01/01/52	03/29/89
3780	2	01/01/64	01/31/89
3781	2	01/01/64	01/31/89
3782	2	01/01/73	01/31/89
3783	2	01/01/82	01/31/89
3784	2	01/01/71	02/02/89
3785	2	01/01/71	02/03/89

Number	Sex	Date of Birth	Date of Entry
3792	2	01/01/85	02/09/89
3793	1	01/01/73	02/08/89
3794	2	01/01/54	02/10/89
3795	2	01/01/69	02/16/89
3796	2	01/01/86	02/16/89
3797	2	01/01/74	02/16/89
3798	2	01/01/74	02/16/89
3799	2	01/01/77	02/16/89
3800	2	01/01/74	02/16/89
3801	2	01/01/74	02/16/89
3802	2	01/01/79	02/16/89
3803	2	01/01/67	02/16/89
3804	1	01/01/88	02/16/89
3805	2	01/01/52	02/16/89
3806	2	01/01/61	02/16/89
3807	1	01/01/88	02/16/89
3808	2	01/01/88	02/16/89
3809	2	01/01/88	02/16/89
3810	1	01/01/67	02/16/89
3812	2	01/01/65	03/17/89
3815	2	01/01/79	02/21/89
3816	2	01/01/85	02/21/89
3817	1	06/01/88	02/21/89
3818	1	01/01/86	02/21/89
3819	2	01/01/59	02/21/89
3820	1	01/01/81	02/21/89
3822	1	01/01/85	02/24/89
3823	2	01/01/72	02/28/89
3824	1	01/01/74	03/07/89
3825	1	01/01/73	02/28/89
3828	1	01/01/82	03/01/89
3829	1	01/01/61	03/01/89
3830	1	01/01/84	03/01/89
3834	2	01/01/59	03/08/89
3836	2	01/01/73	03/08/89
3837	2	01/01/66	03/08/89
3838	2	01/01/77	03/08/89
3840	2	01/01/74	03/15/89
3841	1	01/01/79	03/15/89
3842	2	01/01/80	03/15/89
3844	1	01/01/71	03/16/89
3845	2	01/01/77	03/14/89
3846	1	01/01/80	03/17/89
3847	1	01/01/69	03/17/89
3849	2	01/01/54	03/21/89
3850	1	01/01/69	03/21/89
3851	1	01/01/79	03/21/89
3854	1	01/01/75	03/30/89
3855	1	01/01/59	03/30/89
3857	2	01/01/64	04/04/89
3860	1	01/01/80	04/06/89
3861	1	01/01/64	04/06/89

Number	Sex	Date of Birth	Date of Entry
3866	2	01/01/84	04/13/89
3867	1*	03/01/89	04/13/89
3869	2	01/01/49	04/12/89
3870	2	01/01/83	04/10/89
3871	2	01/01/85	04/13/89
3872	2	01/01/83	04/13/89
3873	2	01/01/49	04/13/89
3874	2	01/01/49	04/13/89
3876	2	01/01/54	04/17/89
3877	1	01/01/84	04/17/89
3878	1	01/01/64	04/21/89
3879	1	01/01/79	04/13/89
3881	1	01/01/69	04/28/89
3882	2	01/01/77	04/27/89
3884	1	01/01/77	04/21/89
3886	1	01/01/88	05/03/89
3887	1	01/01/59	05/03/89
3888	1	01/01/62	05/03/89
3889	1	01/01/64	05/03/89
3891	2	01/01/67	05/02/89
3892	1	01/01/82	05/04/89
3898	2	09/01/79	08/16/89
3899	2	01/01/44	05/16/89
3900	2	01/01/49	05/17/89
3907	2	01/01/68	05/15/89
3910	1	01/01/85	05/24/89
3911	1	01/01/69	05/24/89
3916	1	01/01/85	06/02/89
3917	2	01/01/88	06/02/89
3918	2	01/01/54	06/02/89
3919	2	01/01/77	06/02/89
3920	2	01/01/59	05/30/89
3921	1	01/01/72	05/30/89
3922	1	01/01/75	05/30/89
3923	1	01/01/86	06/01/89
3924	1	01/01/85	06/01/89
3926	2	10/01/72	06/06/89
3927	1	01/01/65	06/06/89
3928	2	01/01/77	06/06/89
3929	2	01/01/67	06/07/89
3937	1	04/01/70	06/12/89
3938	1	01/01/71	06/12/89
3939	1	01/01/74	06/15/89
3940	1	01/01/69	06/12/89
3941	2	04/01/69	06/12/89
3942	2	01/01/63	06/12/89
3943	2	02/15/80	06/12/89
3944	2	01/01/73	06/12/89
3911	1	01/01/69	05/24/89
3916	1	01/01/85	06/02/89
3917	2	01/01/88	06/02/89
3918	2	01/01/54	06/02/89

Number	Sex	Date of Birth	Date of Entry
3953	2	01/01/51	06/13/89
3954	2	01/01/73	06/22/89
3955	2	01/01/79	06/22/89
3956	2	01/01/52	06/22/89
3957	2	01/01/79	06/22/89
3958	2	01/01/64	06/22/89
3959	2	01/01/67	06/22/89
3960	1	01/01/79	06/22/89
3962	1	01/01/61	06/22/89
3963	1	01/01/57	06/23/89
3965	1	01/01/84	06/23/89
3967	2	01/01/67	06/23/89
3968	1	01/01/63	06/29/89
3969	1	01/01/77	06/29/89
3970	1	01/01/86	06/29/89
3971	1	01/01/81	06/29/89
3972	1	01/01/81	06/29/89
3973	1	01/01/80	06/29/89
3974	1	01/01/69	06/29/89
3975	1	01/01/79	06/29/89
3976	1	01/01/72	06/28/89
3977	1	01/01/85	07/03/89
3979	1	01/01/75	07/06/89
3984	1	01/01/64	07/06/89
3985	2	01/01/76	07/06/89
3987	2	07/02/89	07/02/89
3988	2	01/01/54	07/07/89
3989	1	01/01/64	07/07/89
3990	1	01/01/74	07/13/89
3991	2	01/01/74	07/13/89
3993	1	01/01/79	07/14/89
3994	1	01/01/72	07/13/89
4000	1	07/13/89	07/13/89
4001	2	01/01/69	07/12/89
4002	1	01/01/74	07/19/89
4003	2	01/01/72	07/19/89
4005	1	01/01/83	07/21/89
4006	1	03/01/87	07/24/89
4007	1	01/01/75	07/25/89
4008	1	01/01/77	07/25/89
3972	1	01/01/81	06/29/89
3973	1	01/01/80	06/29/89
3974	1	01/01/69	06/29/89
3975	1	01/01/79	06/29/89
3976	1	01/01/72	06/28/89
3977	1	01/01/85	07/03/89
3979	1	01/01/75	07/06/89
3984	1	01/01/64	07/06/89
3985	2	01/01/76	07/06/89
3987	2	07/02/89	07/02/89
3988	2	01/01/54	07/07/89
3989	1	01/01/64	07/07/89

Number	Sex	Date of Birth	Date of Entry
3994	1	01/01/72	07/13/89
4000	1	07/13/89	07/13/89
4001	2	01/01/69	07/12/89
4002	1	01/01/74	07/19/89
4003	2	01/01/72	07/19/89
4005	1	01/01/83	07/21/89
4006	1	03/01/87	07/24/89
4007	1	01/01/75	07/25/89
4008	1	01/01/77	07/25/89
4015	1	07/31/70	07/27/89
4020	2	01/01/74	08/02/89
4021	1	01/01/81	08/02/89
4023	1	01/01/74	08/01/89
4024	1	01/01/77	08/01/89
4027	1	01/01/74	08/10/89
4028	2	01/01/85	08/10/89
4030	2	01/01/72	08/10/89
4031	2	01/01/79	08/10/89
4032	2	01/01/85	08/10/89
4033	2	01/01/84	08/10/89
4034	2	01/01/88	08/10/89
4035	1	01/01/82	08/10/89
4037	1	01/01/64	08/16/89
4039	2	01/01/85	08/16/89
4044	1	08/11/89	08/11/89
4045	2	09/17/76	08/23/89
4046	1	01/01/80	08/23/89
4047	1	01/01/67	08/23/89
4048	1	01/01/67	08/23/89
4049	1	01/01/77	08/23/89
4050	1	01/01/66	08/23/89
4051	1	01/01/76	08/23/89
4052	1	01/01/81	08/25/89
4053	2	01/01/54	08/25/89
4054	2	01/01/69	08/25/89
4057	1	01/01/84	08/25/89
4061	1	01/01/84	09/01/89
4062	1	01/01/70	09/01/89
4064	2	01/01/83	09/01/89
4065	1	01/01/73	08/30/89
4066	1	01/01/67	08/06/89
4067	1	01/01/79	09/01/89
4071	1	01/01/69	09/15/89
4072	1	01/01/59	09/15/89
4073	1	01/01/67	09/15/89
4074	1	01/01/69	09/15/89
4075	1	06/02/87	09/14/89
4077	1	01/01/64	09/26/89
4078	1	01/01/66	09/26/89
4079	1	01/01/73	09/26/89
4080	2	01/01/71	09/26/89
4081	1	01/01/71	09/26/89

Number	Sex	Date of Birth	Date of Entry
4085	2	01/01/82	09/26/89
4091	1	01/01/85	10/12/89
4092	2	01/01/74	10/12/89
4093	1	01/01/49	10/12/89
4094	1	01/01/49	10/12/89
4095	2	01/01/71	10/11/89
4102	2	01/01/74	10/03/89
4103	2	01/01/49	10/10/89
4106	2	01/01/64	10/18/89
4107	1	10/11/89	10/11/89
4109	2	01/01/78	10/24/89
4110	2	01/01/64	10/23/89
4111	2	01/01/59	10/23/89
4112	1	01/01/67	10/23/89
4113	2	01/01/64	10/25/89
4114	2	01/01/68	10/25/89
4115	2	01/01/64	10/25/89
4118	1	01/01/80	10/31/89
4119	2	01/01/69	10/31/89
4120	2	01/01/66	10/31/89
4121	2	01/01/83	10/31/89
4124	1	01/01/69	10/31/89
4125	1	01/01/68	11/01/89
4128	2	01/01/75	11/09/89
4129	2	01/01/71	11/06/89
4130	1	01/01/67	11/02/89
4133	1	01/01/85	11/16/89
4136	2	01/01/65	11/16/89
4137	2	01/01/67	11/16/89
4138	2	01/01/75	11/16/89
4139	1	01/01/69	11/16/89
4140	2	01/01/70	11/17/89
4141	2	01/01/79	11/17/89
4142	2	01/01/65	11/16/89
4143	1	01/01/69	11/16/89
4144	2	01/01/74	11/17/89
4146	1	01/01/87	11/22/89
4147	1	01/01/85	11/22/89
4148	1	01/01/84	11/22/89
4149	2	06/14/69	11/22/89
4150	1	01/01/75	11/17/89
4151	1	01/01/82	11/29/89
4152	1	01/01/77	11/29/89
4153	2	01/01/66	11/29/89
4154	1	01/01/75	11/29/89
4158	2	01/01/61	11/30/89
4159	2	01/01/65	11/30/89
4160	2	01/01/83	11/30/89
4162	1	01/01/61	12/04/89
4163	2	01/01/59	12/04/89
4164	2	01/01/74	12/04/89
4166	1	01/01/63	12/08/89

Number	Sex	Date of Birth	Date of Entry
4172	2	01/01/67	12/08/89
4173	2	01/01/69	12/08/89
4174	1	01/01/73	12/08/89
4177	2	01/01/49	12/08/89
4178	2	01/01/49	12/08/89
4179	2	01/01/55	12/08/89
4180	1	01/01/67	12/08/89
4181	1	01/01/65	12/08/89
4182	2	01/01/74	12/08/89
4183	1	01/01/49	12/08/89
4184	1	01/01/87	12/08/89
4185	1	01/01/86	12/08/89
4186	1	01/01/86	12/08/89
4187	1	01/01/80	12/08/89
4188	2	01/01/86	12/08/89
4189	2	01/01/79	12/08/89
4190	2	01/01/87	12/08/89
4191	2	01/01/67	12/08/89
4192	2	01/01/73	12/08/89
4193	2	01/01/80	12/08/89
4195	1*	01/01/49	12/15/89
4196	2	01/01/64	12/15/89
4197	2	01/01/87	12/15/89
4198	1	01/01/65	12/15/89
4201	2	01/01/77	12/15/89
4202	2	09/01/89	12/15/89
4203	2	01/01/82	12/15/89
4204	1	07/01/89	12/15/89
4205	2	01/01/74	12/15/89
4206	1	10/01/89	12/15/89
4207	2	01/01/64	12/15/89
4208	1	06/01/89	12/15/89
4209	1	01/01/73	12/15/89
4210	2	01/01/77	12/15/89
4211	1	01/01/69	12/15/89
4212	1	01/01/71	12/15/89
4213	2	01/01/84	12/15/89
4214	2	01/01/89	12/15/89
4215	2	01/01/85	12/15/89
4216	2	01/01/85	12/15/89
4217	1	01/01/82	12/15/89
4219	1	01/01/86	12/15/89
4220	1	01/01/84	12/15/89
4221	2	01/01/60	12/19/89
4222	1	01/01/72	12/19/89
4223	1	01/01/78	12/19/89
4224	1	01/01/78	12/19/89
4225	1	01/01/73	12/19/89
4226	2	01/16/87	12/19/89
4227	1	01/01/80	12/19/89
4228	2	01/01/72	12/19/89
4229	2	01/01/77	12/19/89

Number	Sex	Date of Birth	Date of Entry
4233	2	01/01/84	12/21/89
4234	2	01/01/75	12/21/89
4263	2	07/24/88	01/22/90
4264	2	07/23/88	01/22/90
7003	1	01/01/85	10/21/88
7027	2	04/01/75	08/09/88
7028	2*	01/01/66	08/09/88

APPENDIX II

CASE POPULATION DATA

Key: **Sex 1; male (gelding)**

Sex 2; female (jenny)

***; Dead donkey (See Appendix IV)**

Number	Sex	Date of Birth	Date of Entry	Date Lesion Observed
128	1*	01/01/52	24/07/74	14/07/85
167	1	01/01/72	10/09/74	12/01/76
248	2	01/01/66	01/01/75	19/04/83
344	2	01/01/62	18/03/76	15/07/86
422	1*	01/01/72	21/04/77	01/10/84
425	2	01/01/65	21/04/77	30/05/85
429	2	01/01/75	08/05/77	01/11/83
457	1*	01/01/47	02/09/77	12/04/78
514	2	01/05/78	03/05/78	09/07/85
543	2	01/07/78	18/07/78	23/01/87
556	1	01/01/77	08/09/78	07/06/83
560	2	01/01/70	14/09/78	15/10/87
676	1*	01/01/49	17/07/79	16/05/84
730	2	01/01/80	24/01/80	17/01/84
757	2	01/01/77	11/03/77	24/03/82
760	2	01/01/78	11/03/80	11/09/85
835	2	01/01/76	29/04/80	14/07/85
863	2	01/01/60	12/10/80	06/08/88
868	1	01/01/79	29/10/80	05/08/83
876	2	01/03/80	06/11/80	14/12/88
886	2	01/01/78	18/11/80	23/07/83
902	2	01/01/72	01/12/80	01/10/81
978	1	01/01/67	10/04/81	30/07/85
991	2*	01/01/50	24/04/81	30/07/85
1070	1	01/04/79	27/08/81	09/02/84
1096	2	01/01/63	13/10/81	25/07/86
1120	1	01/09/80	04/11/81	05/06/86
1134	2*	01/01/65	30/11/81	06/06/83
1166	1*	01/01/67	21/08/82	15/10/84
1217	1	01/01/75	24/03/82	01/05/86
1228	1	01/01/81	02/04/82	01/08/86
1236	1	01/06/80	16/04/82	10/05/83
1237	1	01/11/80	16/04/82	31/07/86
1296	2*	01/01/80	07/07/82	06/06/84
1301	1	01/03/81	07/07/82	12/07/85
1336	1	01/07/81	02/09/82	06/04/84
1338	2*	01/01/80	03/09/82	07/03/85
1354	1	01/01/76	12/10/82	13/01/88
1370	2	01/01/70	10/11/82	03/07/85
1493	2	01/01/81	12/04/83	16/07/86
1519	1	01/01/75	10/05/83	30/07/86
1657	1	01/01/80	21/10/83	10/07/87
1708	2	01/01/79	09/12/83	19/10/84
1727	1	01/01/81	13/01/84	15/07/86
1734	1	01/01/82	27/01/84	28/02/85
1750	1*	01/01/80	10/02/84	24/06/85
1762	1	01/01/64	09/03/84	17/11/89
1789	2	01/02/73	05/04/84	09/03/88
1826	1	01/01/83	11/05/84	04/09/86
1842	2	01/01/74	25/05/84	17/09/86
1998	1	01/01/84	30/11/84	18/02/87
2070	1	01/01/82	13/02/85	02/07/87

Number	Sex	Date of Birth	Date of Entry	Date Lesion Observed
2148	1	01/01/83	04/04/85	13/11/86
2154	2	01/11/78	10/04/85	14/02/89
2245	1	01/01/84	12/06/85	14/06/89
2344	1	01/01/79	23/09/85	13/04/86
2400	2	01/01/76	24/10/85	05/02/87
2428	2	18/10/81	18/10/85	04/09/86
2435	2	01/01/82	14/11/85	22/04/87
2439	2	01/01/80	14/11/85	08/11/89
2515	1	01/06/79	13/01/86	21/11/88
2627	1	01/01/77	09/04/86	12/09/88
2712	2	01/01/82	25/06/86	30/10/89
2735	1	01/01/85	18/07/86	01/06/87
2817	1	01/01/79	01/10/86	06/06/88
2932	1	01/01/77	21/01/87	19/01/90
3008	2	01/01/81	14/04/87	14/08/89
3037	1	01/01/69	08/05/87	13/12/88
3051	1	01/01/84	22/05/87	29/09/89
3075	2	01/01/85	08/06/87	25/08/88
3346	1	01/01/86	29/03/88	02/03/89
3408	1	01/01/86	23/03/88	14/02/89
3470	1	01/01/87	27/05/88	03/11/89
3696	1	01/01/87	01/12/88	20/09/89
3700	1	01/01/87	01/12/88	02/02/90
3914	1	01/01/70	24/05/89	07/03/90
3966	1	01/01/85	23/06/89	16/05/90

APPENDIX III

PRE-ENTRY POPULATION DATA

Key: **Sex 1; male (gelding)**

Sex 2; female (jenny)

Number	Sex	Date of Birth	Date of Entry
723	2	01/01/56	08/01/80
992	1	01/01/78	24/04/81
1084	1	01/01/75	22/09/81
1277	1	01/01/78	11/06/82
1310	2	01/01/66	22/07/82
1415	2	01/01/72	21/12/82
1911	2	01/01/72	15/09/84
2000	1	01/01/54	30/11/84
2204	1	01/01/70	12/06/85
2212	1	01/01/80	14/05/85
2353	2	01/01/83	27/09/85
2369	1	01/01/72	09/10/85
2406	1	01/01/70	29/10/85
2522	2	01/01/84	17/01/86
2529	1	01/01/74	23/01/86
2535	2	01/01/82	03/01/86
2562	2	01/01/61	21/02/86
2687	1	01/01/82	27/05/86
2732	1	01/01/82	18/07/86
3151	1	01/01/81	22/07/87
3157	1	01/01/80	20/01/82
3230	1	01/01/77	16/10/87
3259	2	01/01/85	25/10/87
3384	1	01/01/85	04/03/88
3399	1	01/01/84	15/03/88
3459	1	01/01/82	18/05/88
3579	2	01/01/77	18/08/88
3585	1	01/01/68	25/08/88
3590	1	01/01/84	31/08/88
3626	2	01/01/63	29/09/88
3675	1	01/10/82	09/11/88
3858	1	01/01/49	06/04/89
3906	1	01/01/85	15/05/89
3913	1	01/01/86	19/05/89
3915	1	01/01/83	02/06/89
3930	2	01/01/83	07/06/89
4123	2	01/01/59	31/10/89
4126	1	01/01/82	07/11/89
4218	1	01/01/87	15/12/89
4365	1	01/01/60	08/04/90
4408	1	01/01/86	21/05/90
4417	1	01/01/87	25/05/90
4442	1	01/01/85	08/06/90

APPENDIX IV

DEAD POPULATION DATA

Number	Date of Birth	Age at Death
1	01/01/63	15
3	01/01/69	5
6	01/01/63	11
8	01/01/60	27
9	01/01/53	33
10	01/01/53	34
11	01/01/69	6
15	01/01/69	20
16	01/01/66	23
18	01/01/68	8
22	05/01/72	4
25	01/01/63	23
28	01/01/70	7
34	01/01/70	20
35	01/01/70	17
39	01/01/63	14
42	01/01/47	31
43	01/01/40	35
44	01/01/59	19
47	01/01/38	40
48	01/01/71	15
50	01/01/73	16
52	01/01/58	17
53	01/01/63	18
58	02/01/74	1
60	01/01/62	16
63	01/01/67	11
64	01/01/60	21
65	01/01/59	25
66	04/01/73	1
67	01/01/66	20
69	01/01/53	25
73	01/01/69	12
74	01/01/71	20
78	01/01/65	20
83	01/01/64	25
84	01/01/66	23
85	01/01/70	11
89	01/01/70	9
90	01/01/60	23
93	01/01/70	8
94	01/01/65	13
95	01/01/54	31
96	01/01/73	16
97	01/01/70	5
99	01/01/67	9
100	01/01/69	7
101	01/01/44	35
102	01/01/54	27
103	01/01/60	18
105	01/01/58	19
106	01/01/73	7

Number	Date of Birth	Age at Death
113	01/01/70	8
114	01/01/70	8
120	01/01/68	9
121	01/01/40	48
122	01/01/66	17
134	01/01/65	13
136	01/01/57	31
138	01/01/60	23
144	01/01/60	23
145	01/01/69	6
148	01/01/64	14
152	01/01/69	7
153	01/01/54	31
155	01/01/68	16
156	01/01/49	34
157	01/01/40	36
161	01/01/69	7
162	01/01/54	29
163	01/01/44	35
169	01/01/60	16
171	01/01/67	21
174	01/01/68	8
177	01/01/60	22
178	01/01/67	16
181	01/01/71	6
183	01/01/25	54
186	11/01/74	1
187	01/01/64	24
188	01/01/62	17
189	01/01/70	6
190	01/01/60	22
193	01/01/54	25
194	01/01/55	27
195	01/01/64	16
196	01/01/64	23
198	01/01/67	9
202	01/01/70	7
204	01/01/68	8
205	01/01/72	18
207	01/01/71	19
208	01/01/70	13
209	01/01/55	27
210	01/01/70	13
211	01/01/73	10
212	01/01/70	9
217	01/01/74	17
223	01/01/72	14
226	01/01/70	12
228	05/01/63	25
229	01/01/50	28
230	01/01/68	9
232	01/01/63	20

Number	Date of Birth	Age at Death
242	01/01/63	14
246	01/01/69	13
250	01/01/75	15
251	01/01/65	18
255	01/01/70	18
260	01/01/70	6
262	01/01/68	16
272	01/01/70	10
276	01/01/45	37
277	01/01/55	28
279	01/01/65	12
281	01/01/55	28
282	01/01/45	40
283	07/01/75	8
286	01/01/55	25
287	01/01/74	11
288	01/01/69	8
290	01/01/60	30
297	01/01/71	12
298	01/01/55	26
299	01/01/45	35
302	01/01/63	20
304	01/01/70	6
306	01/01/59	17
307	01/01/61	18
311	01/01/66	13
312	01/01/57	25
314	01/01/70	20
320	01/01/70	14
329	01/01/73	6
335	01/01/60	22
343	01/01/50	27
345	01/01/70	13
350	01/01/70	13
351	01/01/66	22
368	01/01/60	29
369	01/01/74	12
376	01/01/56	30
377	01/01/73	6
378	01/01/69	16
381	01/01/69	14
382	01/01/56	26
383	01/01/70	7
386	01/01/56	24
387	01/01/46	37
388	01/01/66	20
389	01/01/72	15
393	01/01/57	24
397	01/01/65	14
401	01/01/70	8
407	02/01/77	1
411	01/01/72	11

Number	Date of Birth	Age at Death
435	01/01/65	17
438	01/01/48	36
439	01/01/62	28
441	01/01/59	19
443	01/01/63	20
444	04/01/75	8
446	07/01/77	13
447	01/01/67	22
449	01/01/50	38
451	01/01/71	8
454	01/01/42	43
455	01/01/59	26
456	01/01/47	57
458	01/01/52	31
459	01/01/55	35
460	01/01/57	29
461	01/01/67	21
465	01/01/75	6
477	01/01/63	24
478	01/01/73	11
483	01/01/47	33
492	01/01/66	13
493	01/01/58	21
494	01/01/53	31
497	01/01/68	21
498	01/01/76	3
502	01/01/64	23
505	01/01/72	12
507	01/01/62	21
512	01/01/65	15
517	01/01/52	37
518	01/01/47	42
519	01/01/43	42
520	01/01/43	36
522	01/01/48	36
524	01/01/51	37
527	01/01/48	34
532	01/01/63	21
534	01/01/73	6
538	01/01/58	23
539	01/01/58	21
546	01/01/68	19
547	01/01/48	41
548	01/01/72	11
552	01/01/62	23
553	01/01/72	9
555	01/01/73	6
562	01/01/55	26
572	01/01/63	16
573	01/01/71	8
577	01/01/62	23
585	01/01/65	22

Number	Date of Birth	Age at Death
598	01/01/73	17
602	01/01/66	17
604	01/01/58	31
606	01/01/70	20
608	01/01/77	4
609	01/01/69	17
610	01/01/56	33
613	01/01/59	21
615	01/01/62	19
617	01/01/49	34
624	01/01/69	13
625	01/01/69	13
626	03/01/79	10
627	01/01/62	21
631	01/01/29	51
632	01/01/69	16
635	01/01/44	36
639	01/01/71	13
644	01/01/49	37
645	01/01/73	11
651	05/01/79	3
654	01/01/39	45
659	01/01/65	24
664	01/01/61	24
670	01/01/70	10
673	01/01/59	22
675	01/01/49	37
682	01/01/63	20
685	01/01/72	9
688	01/01/49	32
689	01/01/61	29
690	05/01/76	13
693	01/01/68	13
704	01/01/74	15
705	01/01/59	25
707	01/01/39	42
708	01/01/63	26
711	01/01/64	22
712	01/01/69	12
718	01/01/65	16
720	01/01/49	41
732	01/01/77	4
737	01/01/69	20
742	01/01/72	14
743	01/01/58	27
745	01/01/63	23
750	01/01/45	36
753	01/01/50	34
755	01/01/66	19
758	01/01/62	23
765	01/01/50	38
767	01/01/55	32

Number	Date of Birth	Age at Death
794	01/01/73	10
798	01/01/72	18
808	01/01/71	11
813	01/01/65	22
814	01/01/76	5
818	01/01/70	13
822	01/01/73	8
823	01/01/55	30
827	01/01/60	25
833	01/01/70	15
834	09/01/80	4
836	01/01/70	20
844	01/01/55	35
847	01/01/55	35
850	01/01/56	31
852	01/01/62	28
857	01/01/73	12
871	01/01/60	23
882	01/01/68	16
894	01/01/74	10
895	01/01/58	30
906	01/01/63	22
907	01/01/50	32
909	01/01/74	15
920	01/01/65	18
931	01/01/72	11
933	01/01/71	19
938	01/01/79	10
943	01/01/77	12
955	01/01/60	27
956	01/01/73	17
960	01/01/41	44
968	01/01/65	25
970	01/01/73	14
971	01/01/71	11
972	01/01/73	9
982	01/01/50	35
985	01/01/64	24
986	01/01/56	27
1000	01/01/70	20
1001	01/01/70	20
1007	01/01/61	29
1008	01/01/59	30
1009	01/01/56	28
1015	01/01/71	12
1019	05/01/81	2
1020	01/01/60	24
1021	01/01/60	22
1023	01/01/71	19
1036	08/01/79	11
1045	01/01/72	12
1048	01/01/74	16

Number	Date of Birth	Age at Death
1065	01/01/74	16
1068	01/01/47	42
1069	01/01/75	13
1076	01/01/66	24
1077	01/01/72	13
1078	01/01/75	10
1079	01/01/72	11
1082	01/01/56	30
1083	01/01/60	26
1088	01/01/69	14
1089	01/01/56	30
1090	01/01/56	29
1106	01/01/51	35
1111	01/01/69	16
1124	01/01/62	28
1126	01/01/70	18
1127	01/01/73	10
1129	06/01/75	14
1135	01/01/65	25
1136	01/01/71	13
1141	01/01/50	33
1142	01/01/61	26
1145	01/01/71	18
1148	01/01/57	31
1151	01/01/72	15
1161	01/01/81	9
1162	01/01/78	12
1178	01/01/65	25
1181	01/01/57	31
1182	01/01/55	35
1185	01/01/66	19
1191	01/01/75	12
1196	01/01/73	17
1198	01/01/70	16
1202	01/01/67	20
1206	10/01/80	6
1218	01/01/60	28
1232	04/01/82	4
1234	01/01/67	20
1240	01/01/48	38
1243	01/01/76	10
1251	01/01/53	31
1260	01/01/65	19
1270	01/01/57	29
1272	01/01/75	14
1287	09/01/74	14
1290	01/01/57	32
1295	01/01/79	6
1297	05/01/82	7
1299	01/01/40	43
1299	01/01/40	43
1308	06/01/72	18

Number	Date of Birth	Age at Death
1346	01/01/69	17
1353	01/01/68	16
1360	01/01/70	16
1385	01/01/72	16
1391	01/01/70	20
1397	01/01/69	20
1398	01/01/68	16
1399	01/01/69	16
1400	01/01/42	46
1401	01/01/68	20
1414	01/01/69	15
1422	01/01/63	24
1433	01/01/75	13
1436	01/01/45	39
1441	01/01/63	27
1442	01/01/74	16
1447	01/01/68	20
1448	01/01/63	24
1454	01/01/71	13
1461	01/01/63	22
1467	01/01/70	17
1471	01/01/53	36
1475	01/01/79	11
1480	06/30/82	5
1483	01/01/53	36
1501	01/01/67	22
1504	01/01/63	27
1507	01/01/73	17
1511	01/01/73	15
1514	01/01/58	32
1520	01/01/71	16
1529	01/01/63	22
1536	01/01/48	38
1539	01/01/43	42
1541	01/01/60	27
1546	01/01/63	22
1547	01/01/73	11
1555	01/01/66	23
1564	01/01/63	27
1568	01/01/79	6
1574	01/01/58	27
1591	01/01/68	18
1616	01/01/69	15
1622	01/01/79	11
1659	01/01/78	7
1660	01/01/70	20
1665	01/01/53	37
1668	01/01/63	22
1671	01/01/70	19
1680	01/01/43	42
1691	01/01/70	19
1694	01/01/71	15

Number	Date of Birth	Age at Death
1758	01/01/54	36
1768	01/01/71	17
1776	01/01/68	22
1784	01/01/71	17
1806	07/01/71	16
1810	01/01/50	39
1818	01/01/78	7
1822	01/01/39	47
1835	01/01/79	6
1836	01/01/79	6
1851	01/01/49	37
1872	01/01/77	8
1873	01/01/70	19
1891	01/01/70	20
1915	01/01/64	24
1922	01/01/80	9
1927	01/01/50	37
1928	01/01/64	24
1938	01/01/69	20
1945	01/01/60	27
1966	01/01/59	29
1967	01/01/60	26
1979	01/01/68	21
1981	01/01/70	16
1982	01/01/70	19
1985	01/01/71	15
1987	01/01/64	22
1993	01/01/67	23
1995	01/01/70	16
2003	01/01/74	14
2005	01/01/69	18
2010	01/01/61	26
2011	01/01/65	23
2017	01/01/70	16
2025	01/01/70	17
2027	01/01/64	22
2031	01/01/81	7
2043	01/01/60	29
2044	01/01/82	4
2050	01/01/81	5
2054	05/01/72	17
2055	06/01/69	18
2059	01/01/57	30
2061	01/01/67	23
2063	01/01/60	28
2067	01/01/70	20
2086	01/01/65	25
2093	01/01/62	26
2115	01/01/70	16
2126	01/01/75	11
2156	01/01/72	15
2174	01/01/65	23

Number	Date of Birth	Age at Death
2205	01/01/70	20
2232	01/01/73	14
2252	01/01/76	10
2252	01/01/76	10
2284	01/01/67	23
2303	01/01/67	20
2306	01/01/65	23
2307	01/01/70	18
2311	01/01/69	20
2315	01/01/64	23
2330	01/01/82	7
2336	09/01/65	23
2337	09/01/60	29
2342	01/01/65	21
2352	01/01/45	41
2354	01/01/55	33
2358	01/01/74	15
2362	01/01/55	33
2368	01/01/50	36
2386	01/01/74	12
2392	01/01/77	9
2442	01/01/73	15
2444	01/01/73	14
2445	01/01/62	28
2453	01/01/60	30
2467	01/01/69	19
2483	01/01/60	29
2532	01/01/46	42
2547	01/01/66	22
2556	01/01/68	21
2565	01/01/58	30
2593	01/01/58	31
2636	01/01/69	20
2640	01/01/60	29
2671	01/01/46	42
2672	01/01/66	23
2695	01/01/71	17
2721	01/01/46	42
2722	01/01/46	43
2731	01/01/60	28
2761	01/01/49	38
2786	01/01/61	28
2825	01/01/62	28
2838	10/01/66	24
2841	01/01/82	7
2845	01/01/61	27
2868	01/01/51	37
2871	01/01/61	26
2884	01/01/61	29
2888	01/01/70	20
2892	01/01/71	17
2893	01/01/56	31

Number	Date of Birth	Age at Death
2916	01/01/61	26
2921	01/01/61	27
2922	01/01/61	27
2923	01/01/59	29
2924	01/01/47	41
2925	01/01/51	37
2938	01/01/60	29
2944	01/01/52	36
2962	01/01/47	41
2963	01/01/47	43
2970	01/01/52	38
3064	01/01/50	40
3071	01/01/76	14
3080	01/01/67	22
3083	01/01/52	37
3085	01/01/67	22
3091	01/01/67	21
3093	01/01/51	37
3097	01/01/62	27
3107	01/01/80	8
3111	01/01/70	18
3113	01/01/85	3
3157	01/01/60	30
3241	01/01/57	31
3257	01/01/65	24
3269	01/01/77	13
3270	01/01/62	27
3278	01/01/47	43
3285	01/01/52	37
3322	01/01/53	36
3369	01/01/53	37
3373	01/01/48	41
3454	01/01/48	42
3480	01/01/56	33
3492	01/01/58	31
3505	01/01/76	13
3519	01/01/76	13
3542	01/01/63	26
3581	01/01/61	28
3624	01/01/48	41
3634	01/01/63	26
3638	01/01/80	10
3639	01/01/48	42
3645	01/01/48	41
3649	01/01/82	8
3663	01/01/63	26
3689	01/01/76	13
3690	01/01/62	27
3693	01/01/48	42
3867	03/01/89	1
4195	01/01/49	41
7028	01/01/66	24

Number	Date of Birth	Age at Death
676	01/01/49	36
991	01/01/50	37
1134	01/01/65	21
1166	01/01/67	20
1296	01/01/80	9
1338	01/01/80	8
1750	01/01/80	7

APPENDIX V

LIST OF SUPPLIERS

Amersham International plc,
UK Sales Office,
Lincoln Place,
Green End,
Aylesbury,
Buckinghamshire,
HP20 2TP.

BDH Chemicals,
Poole,
Dorset,
BH15 1TD.

Beckman Instruments UK Ltd.,
Sands Industrial Estate,
High Wycombe,
Buckinghamshire,
HP12 4JL.

Biorad Laboratories,
Biorad House,
Maylands Avenue,
Hemel Hempstead,
HP2 7TD.

Boehringer Mannheim Corporation,
Bell Lane,
Lewes,
East Sussex,
BN7 1LG.

Gibco BRL,
Life Technologies Ltd.,
PO Box 35,
Renfrew Road,
Paisley,
PA3 4EF.

ILS Ltd.,
14-15, Newbury Street
London,
EC1A 7HU.

Perkin Elmer Ltd.,

Pharmacia, Ltd.,
Pharmacia LKG Biotechnology Division,
Midsummer Boulevard,
Central Milton Keynes,
Buckinghamshire,
MK9 3HP.

Scotlab Ltd.,
Kirkshaws Road,
Coatbridge,
Strathclyde,
ML5 8AD.

Smithkline Beecham Ltd.,
Walton Oaks,
Dorking Road,
Tadworth,
Surrey,
KT20 7NT.

Stratagene Ltd.,
Cambridge Inovation Centre,
Unit 140,
Cambridge Science Park
Cambridge,
C13 4GF.

Sigma Chemical Company
Fancy Road,
Poole,
Dorset,
BH17 7NH.

United States Biochemical Corporation,
PO Box 22400,
Cleveland
Ohio 44122,
USA.

Whatman LabSales Ltd.,
St. Leonard's Road,
20/20, Maidstone
Kent,
ME16 OLS.

REFERENCES

REFERENCES

Adams, R., Calderwood-Mays, M.B. and Peyton, L.C. (1988) Excision of cutaneous tumours in the horse using histologic guidance. *Veterinary Surgery,* **17**, 241-245.

Almeida, J.D., Howatson, A.F. and Williams, M.G. (1962) Electron microscope study of human warts: Sites of virus production and nature of the inclusion bodies. *Journal of Investigative Dermatology*, **38**, 37-345.

Amtmann, E., Muller, H. and Sauer, G. (1980) Equine connective tissue tumours contain unintegrated bovine papilloma virus DNA. *Journal of Virology,* **35**, 962-964.

Amtmann, E. and Sauer, G. (1982) Activation of nonexpressed bovine papillomavirus genomes by tumour promoters. *Nature,* **296,** 675-677.

Amtmann, E., Volm, M. and Wayss, K. (1984) Tumour induction in the rodent *Mastomys natalensis* by activation of endogenous papilloma virus genomes. *Nature,* **308**, 5291-292.

Angelos, J.A. (1990) Equine sarcoid; Characterisation of genetic susceptibility and etiology. Masters Thesis, University of Cornell, Ithaca, USA.

Angelos, J.A., Oppenheim, Y., Rebhun, W., Mohammed, H. and Antczak, D.F. (1988) Evaluation of breed as a risk factor for sarcoid and uveitis in horses. *Animal Genetics,* **19**, 417-425.

Angelos, J.A., Marti, E., Lazary, S. and Carmichael, L.E. (1991) Characterisation of BPV-like DNA in equine sarcoids. *Archives of Virology,* **119**, 95-109.

Ashley, D.S.B. (1990) Introduction. In: *Evans' Histological Appearances of Tumours.* Fourth Edition. Edinburgh: Churchill Livingston, pp1-10.

Baker, J.R., and Leyland, A. (1975) Histological survey of tumours of the horse, with particular reference to those of the skin. *Veterinary Record,* **96**, 419-422

Barbet, J.L., Baxter, G.M. and McMullan, W.C. (1991) Diseases of the Skin. In: *Equine Medicine and Surgery, Volume II.* Fourth Edition. (Colahan, P.T., Mayhew, I.G., Merritt, A.M. and Moore, J.N. eds.). California: American Veterinary Publications, Inc., pp1718.

Barbosa, M.S., Edmonds, C., Fisher, C., Schiller, J.T., Lowy, D.R. and Vousden, K.H. (1990) The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. *EMBO Journal*, **9**, 153-160.

Barr, B.B.B., Benton, E.C., McLaren, K., Bunney, H., Smith, I.W., Blessing, K. and Hunter, J.A.A. (1989) Human papillomavirus infection and skin cancer in renal allograft recipients. *Lancet*, **i**, 124-128.

Bast, R.C., Zbor, B. and Boros, T. (1974) BCG and Cancer. *New England Journal of Medicine,* **290**, 1413-1419, 1458-1468.

Bendixen, H.J. (1965) Bovine enzootic leukosis. *Advances in Veterinary Science,* **10**,129-134.

Benton, C. Shahidullah, H. and Hunter, J.A.A. (1992) Human papillomavirus in the immunosuppressed. *Papillomavirus Report*, **3**, 23-26.

Bertone, A.L. and McClure, J.J. (1990) Therapy for sarcoids. *Compendium on Continuing Education for the Practising Veterinarian*, **12**, 262-265.

Birnboim, H.C. and Doily, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research,* **7**, 1513-1523.

Bistner, S.I. (1974) Medullo-epithelioma of the iris and ciliary body in the horse. *Cornell Veterinarian*, **64**, 588-595.

Bistner, S., Campbell, R.J., Shaw, D., Leininger, J.R. and Gholbrial, H.K. (1983)

Broker, T.R. and Botchan, M. (1986) Papillomaviruses: Retrospectives and prospectives, *Cancer Cells* **4**, 17-36.

Brostrom, H., Bredberg-Raden, U., England, J., Obel, N. and Perlmann, P., (1979) Cell mediated immunity in horses with sarcoid tumours against sarcoid cells *in vitro. American Journal of Veterinary Research,* **40**, 1701- 1706.

Brostrom, H., Fahlbrink, E., Dubath, M.L. and Lazary, S. (1988) Association between equine leucocyte antigens (ELA) and equine sarcoid tumours in the population of Swedish halfbreds and some of their families. *Veterinary Immunology and Immunopathology,* **19**, 215-223.

Brown, P.J. and Holt, P.E. (1985) Primary renal cell carcinoma in four horses. *Equine Veterinary Journal,* **17**, 473-477.

Brown, M.P. (1983) Surgical treatment of equine sarcoid. In: Current Therapy in Equine Medicine (Robinson, N.E. ed.) Philadelphia: W.B. Saunders Co., pp537-539.

Cadeac, C. (1901) Sur la transmission experimentales des papillomes des diverses especes. *Bulletin de la Societe Scientifique Veterinaire de Lyons,* **4**, 333-341.

Case, R.A.M., Hosker, M.E., McDonald, D.B. and Pearson, J.T. (1954) Tumours in the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British Chemical Industry. Part 1. The role of aniline, benzidine, alpha napthylamine and beta napthylamine. *British Journal of Industrial Medicine,* **11**, 75-83.

Campo, M.S. (1987) Papillomas and cancer in cattle. *Cancer Surveys,* **6**, 39-54.

Campo, M.S. (1992) Cell transformation by animal papillomaviruses. *Journal of General Virology,* **73**, 217-222.

Campo, M.S. and Coggins L.W. (1982) Molecular cloning of bovine papillomavirus genomes and comparison of therir sequence homologies by heteroduplex mapping. *Journal of General Virology,* **63**, 255-264.

Campo M.S. and Jarrett, W.F.H. (1986) Papillomavirus infection in cattle: viral and chemical co-factors in naturally and experimentally induced tumours. In: *Papillomaviruses,* Ciba Foundation Symposium 120, (Evered,D. and Clark, S, eds.). Chichester: John Wiley and Sons, pp117-135.

Campo, M.S., Moar, M.H., Sartirana, M.L., Kennedy, I.M. and Jarrett, W.F.H. (1985) The presence of BPV-4 DNA is not required for progression to, or the maintenance of, the malignant state in cancer of the alimentary canal in cattle. *EMBO Journal*, **4**, 189-1825.

Campo, M.S., Moar, M.H., Laird, H.M. and Jarrett, W.F.H. (1981) Molecular heterogeneity and lesion site specificity of cutaneous bovine papillomaviruses. *Virology,* **113**, 323-335.

Campo, M.S. and Spandidos, D.A. (1983) Molecularly cloned bovine papillomavirus DNA transforms mouse fibroblasts *in vitro. Journal of General Virology,* **64**, 549-557.

Carney, H.C., England, J.J., Hodgkin, E.C. Whitley, H.E., Adkinson, D.L. and Sundberg, J.P. (1990) Papillomavirus infection of aged Persian cats. *Journal of Veterinary Diagnostic Investigation,* **2**, 294-299.

Cheevers, W.P., Fatemi-Nainie, S. and Anderson, L.W. (1986) Spontaneous expression of an endogenous retrovirus by the equine sarcoid-derived MC-1 cell line. *American Journal of Veterinary Research,* **47**, 50-52.

Cheevers, W.P., Roberson, S.M., Brassfield, A.L., Davis, W.C. and Crawford, T.B. (1982) Isolation of a retrovirus from cultured equine sarcoid tumour cells. *American Journal of Veterinary Research,* **43**, 804-806.

Chen, E.Y. and Seeberg, P.H. (1985) Supercoil sequencing. A fast and simple method for sequencing plasmid DNA. *DNA,* **4**, 165-170.

Cheville, N.F. and Olson, C. (1964) Cytology of the canine oral papilloma. *American Journal of Pathology,* **45**, 849-872.

Claas, E.C.J., Melchers, W.J.G., van der Linden, H.C., Lindeman, J. and Quint, W.G.V. (1989) Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the Polymerase Chain Reaction. *American Journal of Pathology,* **135**, 703-709.

Cook, R.H. and Olson, C. (1951) Experimental transmission of cutaneous papilloma of the horse. *American Journal of Pathology,* **21**, 1087-1097.

Cotchin, E. (1977) A general survey of tumours in the horse. *Equine Veterinary Journal,* 9, 16-21.

Cotran, R.S., Kumar, V. and Robbins, S.L. (1989) Neoplasia. In: *Robbins Pathologic Basis of Disease.* Fourth edition (Cotran, R.S., Kumar, V. and Robbins, S.L. eds.). London: W.B. Saunders Company, pp239-307

Cowsert, L.M., Lake, P. and Jenson, A.B. (1987) Topographical and conformational epitopes of bovine papillomavirus type 1 defined by monoclonal antibodies. *Journal of the National Cancer Institute,* **79**, 1053-1057.

Crawford, L.V. (1965) A study of human papillomaviral DNA. *Journal of Molecular Biology,* **13**, 362-372.

Danos, O. and Yaniv, M. (1983) Structure and function of papillomavirus genomes. DNA virus oncogenes and their action. In: *Advances in Viral Oncology,* vol. 3. (Klein, G., ed) New York: Raven Press, pp59-82.

Danos, O., Giri, I., Thierry, F. and Yaniv, M. (1984) Papillomavirus genomes: sequences and consequences. *Journal of Investigative Dermatology,* **83**, 7s-11s.

Davis, C.L. and Kemper H.E. (1936) Common warts (papillomata) in goats. *Journal of the American Veterinary Medical Association,* **88**, 175-179.

Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) Basic Methods in Molecular Biology. New York: Elsevier Science Publishing Co.

Dorn, C.R. and Priester, W.A. (1987) Epidemiology. In: *Veterinary Cancer Medicine.* Second edition. (Theilen, G.H. and Madewell, B,R. eds.). Philadelphia: Lea and Febinger, pp27-52.

Dvoretzky, I., Shober, R., Chattopadhy, S.K. and Lowy D.R. (1980) A quantitative *in vitro* focus forming assay for bovine papilloma virus. *Virology*, **103**, 369-375

Engel, L.W., Heilman, C.A. and Howley, P.M. (1983) Transcriptional organisation of bovine papillomavirus type 1. *Journal of Virology,* **47**, 516-528.

England, J.J, Watson, R.E. and Larson, K.A. (1973) Virus-like particles in an equine sarcoid cell line. American Journal of Veterinary Research, 34, 1601-1603.

Fatemi-Nainie, S., Anderson, L.W. and Cheevers, W.P. (1982) Identification of a transforming retrovirus fom cultured equine dermal fibrosarcoma. *Virology,* **120**, 490-494.

Fatemi-Nainie, S., Anderson, L.W. and Cheevers, W.P. (1984) Culture characteristics and tumorigenicity of the equine sarcoid-derived MC-1 cell line. *American Journal of Veterinary Research*, **45**, 1105-1108.

Favre, M., Breitburd, F., Croissant, O. and Orth, G. (1975) Structural polypeptides of rabbit, bovine and human papillomaviruses. *Journal of Virology,* **15**, 1239-1247.

Favre, M., Jibard, N. and Orth, G. (1982) Restriction mapping and characterisation of the cottontail rabbit papillomavirus genome in transplantable VX2 and VX7 domestic rabbit carcinomas. *Virology,* **119**, 298-309.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry,* **132**, 6-13.

Fenner, F., Bachmann, P.A., Gibbs, E.P.J., Murphy, F.A., Studdert, M.J. and White, D.O. eds. (1987) Papovaviridae. In: *Veterinary Virology.* London:

Ferenczy, A., Bergeron, C. and Richart, R.M. (1989) Human papillomavirus DNA in fomites on objects used for the management of patients with genital human papillomavirus infections. *Obstetrics and Gynecology,* **74**, 950-954.

Finbow, M.E., Pitts, J.D., Goldstein, D.J., Schlegel, R. and Findlay, J.B.C. (1991) The E5 oncoprotein target: a 16kd channel-forming protein with diverse functions. *Molecular Carcinogenesis,* **4**, 441-444.

Fitzwygram, F. (1881) Warts. In: *Horses and Stables,* Second edition. London: Longmans, Green and Co., pp509-510.

Franke, W.W., Appelhans, B., Schmid, E., Freudenstein, C., Osborn, M. and Weber, K. (1979) Identification and characterisation of epithelial cells in mammalian tissues by immunofluorescence microscopy using antibodies to prekeratin. *Differentiation,* **15**, 7-25.

Franke, W.W., Moll, R., Achstaetter, T. and Kuhn C. (1986) Cell typing of epithelia and carcinoma of the female genital tract using cytoskeletal proteins as markers. *Banbury Report*, **21**, 121

Franke, W.W., Schmid, E., Osborn, M. and Weber, K. (1978) Different intermediate-sized filaments distinguished by immunofluorescence microscopy. *Proceedings of the National Academy of Sciences of the USA,* **75**, 5034-5038.

Fretz, P.B. and Barber, S.M. (1980) Prospective analysis of cryosurgery as the sole treatment for equine sarcoids. *Veterinary Clinics of North America: Small Animal Practice,* **10**, 847-859.

Fretz P.B. and Holmberg, D.L. (1980) Sequelae to cryosurgery. *Veterinary Clinics of North America: Small Animal Practice,* **10**, 869-875.

Fulton, R.E., Doane, F.W. and MacPherson, L.W. (1970) The fine structure of equine papillomas and the equine papilloma virus. *Journal of Ultrastructure Research*, **30**, 328-343.

Galloway, D.A. and Jenison, S.A. (1990) Characterisation of the humoral reponse to genital papillomaviruses. *Molecular Biology and Medicine,* **7**, 59-72.

Gerber, H., Dubath, M.-L. and Lazary, S. (1988) Association between predisposition to equine sarcoid and MHC in multiple-case families. In: *Equine Infectious Diseases V.* (Powell, D.G. ed) Lexington: The University Press of Kentucky, pp272-279.

Gettinby, G., Thorpe, J. and Anderson, D. (1989) Construction and use of a clinical database. *Proceedings of the British Cattle Veterinary Association* 4th-5th April, 1989.

Genetzky, R.M., Biwer, R.D. and Meyers, R.K. (1983) Equine sarcoids: Causes, diagnosis and treatment. *Compendium on Continuing Education for the Practising Veterinarian,* **5**, S146-S422.

Gibbs, EP.J., Smale, C.J. and Lawman, M.P.J. (1975) Warts in sheep. *Journal of Comparative Pathology,* **85**, 327-334.

Goldstein, D.J., Finbow, M.E., Andresson, T., McLean, P., Smith, K.T., Bubb, V. and Schlegel, R. (1991) Bovine papillomavirus E5 binds to the 16k component of vacuolar H^+-ATPases, *Nature,* **352**, 347-349.

Gorman, N.T. (1985) Equine sarcoid: time for optimism. *Equine Veterinary Journal,* **17**, 412-414.

Gorra, J.B., Lancaster, W.D., Kurman, R.J. and Jenson, A.B. (1985) Bovine type 1 monoclonal antibodies. *Journal of the National Cancer Institute,* **75**, 121-125

Graffi, A., Bender, E., Schramm, T., Kahn, W. and Schneiders, F. (1969) Induction of transmissible lymphomas in Syrian hamsters by application of DNA from viral hamster papovavirus-induced tumours and by cell-free filtrates from human tumours. *Proceedings of the National Academy of Sciences of the USA,* **64**, 1172-1176.

Groff, D.E. and Lancaster, W.D. (1984) Evidence that integration of virus DNA

Guerrero, E. and Shah, K.V. (1991) Polymerase chain reaction in HPV diagnosis. *Papillomavirus Report,* **2**, 115-118.

Gyllensten, U.B. (1989) PCR and DNA sequencing. *Biotechniques,* **7**, 700-707.

Hamada, M., Oyamada, T., Yoshikawa, H., Yoshikawa, T. and Itakura, C. (1990a) Histopathological development of equine cutaneous papillomas. *Journal of Comparative Pathology,* **102**, 393-403.

Hamada, M., Oymada, T., Yoshikawa, H., Yoshikawa, T. and Itakura, C. (1990b) Keratin expression in equine normal epidermis and cutaneous papillomas using monoclonal antibodies. *Journal of Comparative Pathology*, **102**, 405-420.

Hargis, A.M. (1981) A review of solar induced lesions in domestic animals. *Compendium on Continuing Education for the Practising Veterinarian,* **4**, 287-291.

Head, K.W. (1965) Some data concerning the distribution of skin tumours in domestic animals. In: *Comparative Physiology and Pathology of the Skin.* (Rook, A.J. and Walton, G.S. eds.). Philadelphia: F.A. Davis Company, pp615-636.

Heilman, C.A., Engel, L., Lowy, D.R. and Howley, P.M. (1982) Virus-specific transcription in bovine papillomavirus-transformed mouse cells. *Virology,* **119**, 22-27.

Heinrichs, M., Baumgartner, W. and Capen, C.C. (1990) Immunocytochemical demonstration of proopiomelanocortin-derived peptides in pituitary adenomas of the pars intermedia in horses. *Veterinary Pathology*, **27**, 419-425.

Hepler, D.I. and Leuker, D.C. (1980) Equine sarcoids. *Journal of the American Veterinary Medical Association*, **176**, 388.

Hettle, S.J.H. (1985) Expression of TN1/3 transposase. Ph.D. Thesis, University of Glasgow, Scotland.

Hoffman, K.D., Kainer, R.A. and Shideler, R.K. (1983) Radio-frequency current induced hyperthermia for the treatment of equine sarcoid. *Equine Practice,* **5**, 24-31.

Houlton, J.E.F. (1983) Treatment of periocular equine sarcoids. *Equine Veterinary Journal,* **15** (supplement 2), 117-122.

Hoshimoto, K., Eto, H., Matsumoto, M. and Hori, K. (1983) Anti-keratin monoclonal antibodies: Production, specificities and applications. *Journal of Cutaneous Pathology,* **10**, 529-539.

Howarth, S. (1990) Sarcoids: The story so far. In: *The Veterinary Annual,* 30th issue (Grunsell, C.S.G. and Raw, M.E., eds) London: Butterworth and Co. Ltd., pp145-154.

Howley, P.M. and Schlegel, R. (1988) The human papillomaviruses. *American Journal of Medicine,* **85**, 155-158.

Hultgren, B.D., Schmotzer, W.B., Watrous, B.J., Hedstrom, O.R., Schmitz, J.A., Wagner, P.C., Kaneps, A.J. and Gallacher, J.A. (1987) Nasal-maxillary fibrosarcoma in young horses: A light and electron microscopic study. *Veterinary Pathology*, **24**, 194-196.

Jackson, C. (1936) The incidence and pathology of tumours of domesticated animals in South Africa. *Onderstepoort Journal of Veterinary Science and Animal Industry,* **6**, 1-460.

Jackson, M.E., Pennie, W.D., McCaffery, R.E., Smith, K.T., Grindlay, G.J. and Campo, M.S. (1991) The B subgroup bovine papillomaviruses lack an identifiable E6 open reading frame. *Molecular Carcinogenesis,* **4**, 382-387.

Jaggar, R.T., Pennie, W.D., Smith, K.T., Jackson, M.E. and Campo, M.S. (1990) Cooperation between bovine papillomavirus type 4 and *ras* in the morphological transformation of primary bovine fibroblasts. *Journal of General Virology,* **71**, 3041-3046.

Jarrett, W.F.H. (1985) The natural history of bovine papillomavirus infections. In: *Advances in Viral Oncology,* Volume 5 (Klein, G. ed.). NewYork: Raven Press, pp83-101.

Jarrett, W.F.H., Campo, M.S., Moar, M.H., McNeil, P.M., Laird, H.M., O'Neil, B.W. and Murphy, J. (1980) Papilloma viruses in benign and malignant tumours of cattle. In: *Viruses in Naturally-occuring Cancers.* (Essex, M., Todaro, G. and zur Hausen, H, eds.) New York: Cold Spring Harbour Laboratory Press, pp1043-1428.

Jarrett, W.F.H., Crawford, E.M., Martin, W.B. and Davie, F. (1964) A virus-like particle associated with leukaemia (lymphosarcoma). *Nature,* **202**, 567-568.

Jarrett, W.F.H., Murphy, J., O'Neil, B.W. and Laird, H.M. (1978) Virus-induced papillomas of the alimentary tract of cattle. *International Journal of Cancer,* **22**, 323-328.

Jarrett, W.F.H., O'Neil, B.W., Gaukroger, J.M., Laird, H.M., Smith, K.T., and Campo, M.S. (1990a) Studies on vaccination against papillomaviruses: A comparison of purified virus, tumour extract and transformed cells in prophylactic vaccination. *Veterinary Record*, **126**, 450-452.

Jarrett, W.F.H., O'Neil, B.W., Gaukroger, J.M., Smith, K.T., Laird, H.M. and Campo, M.S. (1990b) Studies on vaccination against papillomaviruses: the immunity after infection and vaccination with bovine papillomaviruses of different types. *Veterinary Record,* **126**, 473-475.

Jarrett, W.F.H., O'Neil, B.W., Gaukroger, J.M., Chandrachud, L.M,, Smith, K.T., Grindlay, G.J., McGarvie, G.M. and Campo, M.S. (1991) Studies on vaccination against papillomaviruses: prophylactic and therapeutic vaccination with recombinant structural proteins. *Virology,* **184**, 33-42

Jenison, S.A., Yu, X.-P., Valentine, J.M. and Galloway, D.A. (1989) Human antibodies react with the epitope of the human papillomavirus type 6b L1 open reading frame which is distinct from the type common epitope. *Journal of Virology,* **63**, 809-818.

Jenson, A.B., Rossenthal, J.D., Olson, C., Pass, F., Lancaster, W.D. and Shah, K.V. (1980) Immunological relatedness of papillomavirus from different species. *Journal of the National Cancer Institute,* **64**, 495-498.

Jin, X.W., Cowsert, L.M., Marshall, D., Reed, D., Pilacinski, W.P., Lim, L. and Jenson, A.B. (1990) Bovine serological response to recombinant BPV-1 major capsid protein vaccine. *Intervirology,* **31**, 345-354.

Johnson, J.E., Beech, J. and Saik, J.E. (1988) Disseminated haemangiosarcoma in a horse. *Journal of the American Veterinary Medical Association*, **193**, 1429-1431.

Joyce, J.R. (1976) Cryosurgical treatment of tumours of horses and cattle. *Journal, of the American Veterinary Medical Association,* **168**, 226-229.

Junge, R.E., Sundberg, J.P. and Lancaster, W.D. (1984) Papillomas and squamous cell carcinomas of horses. *Journal of the American Veterinary Medical Association*, **185**, 656-659.

King, T.C., Priehs, D.R., Gum, G.G. and Miller, T.R. (1991) Therapeutic management of ocular squamous cell carcinoma in the horse: 43 cases (1979-1989). *Equine Veterinary Journal,* **23**, 449-452.

Klein, W.R. (1986) BCG immunotherapy in bovine ocular squamous cell carcinoma and equine sarcoid. Ph.D. Thesis, State University Utrecht, The Netherlands.

Koller, L.D. and Olson, C. (1972) Attempted transmission of warts from man, cattle and horses and of the deer fibroma to selected hosts. *Journal of Investigative Dermatology,* **58**, 366-368.

Krahwinkel, D.J., Merkley, D.F. and Howard, D.R. (1976) Cryosurgical treatment of cancerous and non-cancerous diseases in dogs, horses and cats. *Journal of the American Veterinary Medical Association*, **169**, 201-207.

Laemmeli, U.K. (1970) Cleavage of structural proteins during the assembly of the

Lamberti, C., Morrisey, L.C., Grossman, S.R. and Androphy, E.J. (1990) Transcriptional activation by the papillomavirus E6 zinc finger oncoprotein. *EMBO Journal*, **9**, 1907-1913.

Lancaster, W.D. (1979) Physical maps of bovine papillomavirus type 1 and type 2 genomes. *Journal of Virology*, **32**, 684-687.

Lancaster, W.D. (1981) Apparent lack of integration of bovine papillomavirus DNA in virus-induced equine and bovine tumour cells and virus-transformed mouse cells. *Virology*, **108**, 251-255.

Lancaster, W.D., Olson, C. and Meinke, W. (1977) Bovine papilloma virus: presence of virus-specific DNA sequences in naturally-occurring equine tumours. *Proceedings of the National Academy of Sciences of the USA*, **74**, 524-528.

Lancaster, W.D. and Olson, C. (1980) State of bovine papillomavirus DNA in connective tissue tumours. In : *Viruses in Naturally-occurring Tumours*, vol. 7. (Essex, M., Torado, G and zur Hausen, H. eds) New York: Cold Spring Laboratory Press, pp 223-232.

Lancaster, W.D. and Olson, C. (1982) Animal papillomaviruses. *Microbiology Review*, **46**, 191-195.

Lancaster, W.D., Theilen, G.H. and Olson, C. (1979) Hybridisation of bovine papilloma virus type 1 DNA to DNA from virus induced hamster tumours and naturally occurring equine tumours. *Intervirology*, **11**, 227-233.

Lane, J.G. (1977) The treatment of equine tumours by cryosurgery. *Equine Veterinary Journal*, **9**, 127-133.

Lavach, J.D., Sullins, K.E., Roberts, S.M., Severin, G.A., Wheeler, C. and Lueker, D.C. (1985) BCG treatment of periocular sarcoid. *Equine Veterinary Journal*, **17**, 445-448.

Law, M.-F., Lancaster, W.D. and Howley, P.M. (1979) Conserved polynucleotide

Law, M.-F., Lowy, D.R., Dvoretzky, I. and Howley, P.M. (1981) Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. *Proceedings of the National Academy of Sciences of the* USA, **78**, 2727-2731.

Lazary, S., Gerber, H., Glatt, G. and Straub, R. (1985) Equine leucocyte antigens in sarcoid affected horses. *Equine Veterinary Journal,* **17**, 283-286.

Levi, J.E., Delcelo, R., Alberti, V.N., Torloni, H. and Villa, L.L. (1989) Human papillomavirus DNA in respiratory papillomatosis detected by *in situ* hybridisation and the polymerase chain reaction. *American Journal of Pathology,* **135**, 1179-1184.

Lewis, R.E. (1964) Radon implant therapy of squamous cell carcinoma and equine sarcoid. *American Association of Equine Practitioners.* Proceedings of the Tenth Annual Convention, Denver, Coloado, 217-234.

Li, C.-CH., Shah, K.V., Seth, A. and Gilden, R.V. (1987) Identification of the human papillomavirus type 6b L1 open reading frame in condylomas and corresponding antibodies in human sera. *Journal of Virology,* **61**, 2684-2690.

Lilienfeld, A.M. (1976) *Foundations of Epidemiology.* NewYork: Oxford University Press.

Lowy, D.R., Dvoretzky, I., Shober, R., Law, M.-F., Engel, L. and Howley P.M. (1980) *In vitro* tumorigenic transformation by a defined subgenomic fragment of bovine papillomavirus DNA. *Nature,* **287**, 72-74.

Malison, M.D., Morris, R. and Jones, L.W. (1982) Autogenous vaccine therapy for condyloma acuminatum. A doulble-blind controlled study. *British Journal of Venereal Disease,* **58**, 62-65.

Marsh, L., Erfle, M. and Wykes, E.J. (1984) The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene,* **32**, 481-485.

McMullan, W.C. (1982) The skin. In: *Equine Medicine and Surgery,* Third edition Vol.2 (Mansmann, R.A., Mcallister, E.S. and Pratt, P.W. eds.) Santa Barbara: American Veterinary Publications, pp789- 883.

Meinkoth, J. and Wahl, G. (1984) Hybridisation of nucleic acids immobilised on solid supports. *Analytical Biochemistry,* **138**, 267-284.

Melchers, W.J.G., van den Brule, A.J.C., Walboomers, J., de Bruin, M., Burger, M., Herbrink, P., Meijer, C., Lindeman, J. and Quint, W.J.V. (1989) Increased detection of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared to modified FISH and Southern blot analysis. *Journal of Medical Virology*, **27**, 329-335.

Meneguzzi, M., Cerni, C., Kieny, M.P. and Lathe, R. (1991) Immunisation against human papillomavirus type 16 tumour cells with recombinant vaccinia expressing E6 and E7. *Virology,* **181**, 62-69.

Meredith, D., Elser, A.H., Wolf, B., Soma, L.R., Donawick, W.J. and Lazary, S. (1986) Equine leukocyte antigens: relationships with sarcoid tumours and laminitis in two pure breeds. *Immunogenetics,* **23**, 221-225.

Metcalf, J.W. (1971) Improved technique in sarcoid removal. *Proceedings of the American Association of Equine Practitioners*, **17**, 45-47.

Meyers, C. and Laimins, L.A. (1992) *In vitro* model systems for the study of HPV-induced neoplasia. *Papillomavirus Report*, **3**, 1-3.

Meyers, C., and Wettstein, F.O. (1991) The late region differentially regulates the *in vitro* transformation by cottontail rabbit papillomavirus DNA in different cell types. *Virology,* **181**, 637-646.

Miller, J.M., Miller, L.D. and Olson,C. (1972) Inoculation of calves with particles resembling C-type virus from cultures of bovine lymphosarcoma. *Journal of the National Cancer Institute,* **48**, 423-428.

Moar, M.H., Campo, M.S., Laird, H.M. and Jarrett, W.F.H. (1981a) Persistence of

Moar, M.H., Campo, M.S., Laird, H.M. and Jarrett, W.F.H. (1981b) Unintegrated viral DNA sequences in hamster tumours induced by bovine papillomavirus. *Journal of Virology,* **39**, 945-949.

Montpellier, J., Badens, P. and Dieuzeide, R. (1937) Tumeurs schwaniennes cutanees du mulet. *Revue du Medecine Veterinaire (Toulouse)*, **89**, 216-224.

Montpellier, J., Dieuzeide, R. and Badens, P. (1939) Greffe d'une tumeur schwannienne chez le mulet. *Bulletin de l'Academie Veterinare de France* **12**, 91.

Moore, J.N. and Kintner, L.D. (1976) Recurrent oesophageal obstruction due to squamous cell carcinoma in a horse. *Cornell Veterinarian,* **66**, 590-597.

Moreno-Lopez, J., Pettersson, U., Dinter, Z. and Philipson, L. (1981) Characterisation of a papillomavirus from the Euepoean Elk (EEPV). *Virology,* **112**, 589-595.

Morgan, D.M. and Meinke,W. (1980) Isolation of clones of hamster embryo cells transformed by the bovine papillomavirus. *Current Microbiology,* **3**, 247-251.

Muller, H. and Gissman, L. (1978) *Mastomys natalensis* papillomavirus (MnPV) the causative agent of epithelial proliferations: Characterisation of the virus particle. *Journal of General Virology,* **41**, 315-323.

Mullowney, P.C. (1985) Dermatologic disease of horses. Part IV. Environmental, congenital and neoplastic diseases. *Compendium on Continuing Education for the Practising Veterinarian,* **7**, S22-S33.

Munroe, G.A. (1986) Cryosurgery in the horse. *Equine Veterinary Journal,* **18**, 14-17.

Murphy, J.M., Severin, G.A., Lavach, J.D., Hepler, D.I. and Leuker, (1979) Immunotherapy in ocular equine sarcoid. *Journal of the American Veterinary Medical Association,* **174**, 269-272.

Murray, D.R., Ladds, P.W. and Campbell, R.S.F. (1978) Granulomatous and

Nakai, Y., Lancaster, W.D., Lim, L.Y. and Jenson, A.B. (1986) Monoclonal antibodies to genus- and type-specific papillomavirus structural antigens. *Intervirology,* **25**, 30-37.

Neufield, J.L. (1973) Lymphosarcoma in the horse: a review. *Canadian Veterinary Journal,* **14**, 129-135.

Nuovo, G.J., Darfler, M.M., Impraim, C.C. and Bromley, S.E. (1991) Occurrence of multiple types of papillomavirus in genital tract lesions. *American Journal of Pathology*, **138**, 53-58.

O'Banion, M.K., Reichmann, M.E. and Sundberg, J.P. (1986) Cloning and characterisation of an equine cutaneous papillomavirus. *Virology,* **152**, 100-109.

Olson, C. (1948) Equine sarcoid, a cutaneous neoplasm. *American Journal of Veterinary Research,* **9**, 333-341.

Olson, C. (1972) Cutaneous papillomatosis and sarcoid. In: *Equine Medicine and Surgery: A Text and Reference Work,* Second edition (Catcott, E.J and Smithcors, J.F. eds) Wheaton, American Veterinary Publications, Inc., 71-77.

Olson, C. and Cook, R.H. (1951) Cutaneous sarcoma-like lesions of the horse caused by the agent of bovine papilloma. *Proceedings of the Society for Experimental Biology and Medicine,* **77**, 281-284.

Olson, C., Segre, D. and Skidmore, L. (1960) Further observations on the immunity to bovine cutaneous papillomatosis. *American Journal of Veterinary Research,* **21**, 233-242.

O'Neil, B.W. (1988) Studies on immunity to bovine papillomaviruses. M.Sc. Thesis, University of Glasgow, Scotland.

Orth, G., Jeanteur, P. and Croissant, O. (1971) Evidence for localisation of vegetative viral DNA replication by autoradiographic detection of RNA-DNA hybrids in sections of tumours induced by Shope papilloma virus.

Orth, G. (1987) Epidermodysplasia verruciformis. In: *The Papovaviridae.* The Papillomaviruses (Salzman, N.P. and Howley P.M. eds.). New York: Plenum, pp199-243.

Osterhaus, A.D.M.E., Ellens, D.J. and Horzinek, M.C. (1977) Identification and characterisation of a papillomavirus from birds (Firingillidae). *Intervirology,* **8**, 351-359.

Owen, R.R. and Jagger D.W. (1987) Clinical observation on the use of BCG cell wall fraction for treatment of periocular and other equine sarcoids. *Veterinary Record,* **120**, 548-552.

Page, E.H., Tiffany, L.W. and Russell, H.T. (1967) Use of autogenous equine fibrosarcoma vaccine. *Journal of the American Veterinary Medical Association,* **150**, 177.

Palmer, S.E., (1989) Carbon dioxide laser removal of a verrucose sarcoid from the ear of a horse. *Journal of the American Veterinary Medical Association,* **195**, 1125-1127.

Pascoe, P.J. (1982) Colic in a mare caused by a colonic neurofibroma. *Canadian Veterinary Journal,* **23**, 24-27.

Pascoe, R.R. and Summers, P.M. (1981) Clinical survey of tumorous and tumour-like lesions in horses in southeast Queensland. *Equine Veterinary Journal,* **13**, 235-239.

Petti, L., Nilson, N.A. and DiMaio, D. (1991) Activation of the platelet derived growth factor receptor by the bovine papillomavirus E5 transforming gene. *EMBO Journal,* **10**, 845-855.

Pfister, H. (1984) Biology and biochemistry of papillomaviruses. *Reviews of Physiology, Biochemistry and Pharamacology,* **99**, 111-181.

Pfister, H., Gassenmaier, A., Nurnberger, F. and Stuttgen, G. (1983) Human papilloma virus 5-DNA in a carcinoma of an epidermodysplasia

Pfister, H., Krube, J., Dietrich, W., Iftner, T. and Fuchs, P.G. (1986) Classification of the papillomavirus - mapping the genome. In: *Papillomaviruses,* Ciba Foundation Symposium 120, (Evered,D. and Clark, S, eds.). Chichester: John Wiley and Sons, pp3-22.

Pilacinski, W.P., Glassman, D.L., Krzyzek, R.A., Sadowski, P.L. and Robbins, A.K. (1984) Cloning and expression in *Escherichia coli* of the bovine papillomavirus L1 and L2 open reading frames. *Biotechnology,* 356-360.

Pilacinski, W.P., Glassman, D.L., Glassman, K.F., Reed, D.E., Lum, M.A., Marshall, R.F., Muscoplat, C.C. and Faras, A.J. (1986) Immunisation against bovine papillomavirus infection In: *Papillomaviruses,* Ciba Foundation Symposium 120, (Evered, D. and Clark, S, eds.). Chichester: John Wiley and Sons, pp136-148.

Post, R. and Terpstra, J.I. (1947) De oorzaak en de geneging der papillomatose van het rundvee. *Tijdschrift voor Diergeneeskunde,* **72**, 391-397.

Priester, W.A. (1973) Skin tumours in domestic animals. Data from 12 United States and Canadian Colleges of Veterinary Medicine. *Journal of the National Cancer Institute,* **50**, 457-466.

Priester, W.A. and Mantel, N. (1971) Occurence of tumours in domestic animals. Data from 12 United States and Canadian Colleges of Veterinary Medicine. *Journal of the National Cancer Institute,* **47**, 1333-1344.

Ragland, W.L. (1966) The relationship between bovine papilloma and equine sarcoid. Ph.D. Thesis, Washington State University, USA.

Ragland, W.L., Keown, G.H. and Gorham, J.R. (1966) An epizootic of equine sarcoid. *Nature,* **210**, 1399.

Ragland, W.L., Keown, G.H. and Gorham, J.R. (1970a) Equine sarcoid. *Equine Veterinary Journal,* **2**, 2-11.

Ragland, W.L., McLaughlin, C.A. and Spencer, G.R. (1970b) Attempts to relate

Ragland, W.L. and Spencer, G.R. (1968) Attempts to relate bovine papilloma virus to the cause of equine sarcoid: immunity to bovine papilloma virus. *American Journal of Veterinary Research,* **29**, 1363-1366.

Ragland, W.L. and Spencer, G.R. (1969) Attempts to relate bovine papilloma virus to the cause of equine sarcoid: equidae inoculated with bovine papilloma virus. *American Journal of Veterinary Research,* **30**, 743-751.

Rebhun, W.C. (1987) Immunotherapy for sarcoids. In: *Current Therapy in Equine Medicine-2* (Robinson, N.E. ed.) Philadelphia: W.B. Saunders Co., pp637-639.

Rebhun, W.C. (1990) Sarcoid Workshop, Interlaken, Switzerland, March 1990.

Roberts, W.D. (1970) Experimental treatment of equine sarcoid. *Veterinary Medicine and Small Animal Clinican,* **65**, 67-73.

Robertson, W. (1883) Detailed description of the more common forms of skin diseases in the horse. Class II: Diseases of nutrition including new fomations. In: *A Text Book of the Practice of Equine Medicine.* London: Balliere, Tindall and Cox, p765.

Rous, P. and Beard, J.W. (1935) The progresson to carcinoma of virus induced rabbit papillomatosis (Shope). *American Journal of Pathology,* **45**, 849-872.

Saiki R.K., Gelfand D.H., Stoffel, S., Scharf S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1987) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* **239**, 487-491.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual,* second edition. New York: Cold Spring Harbor Laboratory Press.

Sarcoid Workshop (1990) Interlaken , Switzerland, March 1990.

Scott, D.W. (1988) Neoplastic diseases. In: *Large Animal Dermatology.* Philadelphia: W. B. Saunders Co. pp419-467.

Schneider, J.F., McGlennen, R.C. LaBresh, K.V. Ostrow, R.S., and Faras, A.J. (1991) Rhesus papillomavirus type 1 cooperates with an activated *ras* in transforming primary epithelial rat cells independent of dexamethasone. *Journal of Virology,* **65**, 3354-3358.

Schreier, A.A., Allen, W.P., Laughlin, C. and Gruber, J. (1988) Commentary: Prospects for human papillomavirus vaccines and immunotherapies. *Journal of the National Cancer Institute,* **80**, 896-899.

Schumacher, J., Smith, B.L. and Morgan, S.J. (1988) Osteoma of paranasal sinuses of a horse. *Journal of the American Veterinary Medical Association,* **192**, 1449-1450.

Schwartzmann, S.M., Cantrell, J.L., Ribi E. and Ward, J. (1984) Immunotherapy of equine sarcoid with cell wall skeleton (CWS)-trehalose dimycolate (TDM) biologic. *Equine Practice,* **6**, 13-23.

Segre, D., Olson, C. and Hoerlein, A.B. (1955) Neutralisation of bovine papilloma virus with serum from cattle and horses with experimental papillomas. *American Journal of Veterinary Research*, **16**, 517-520.

Shah, K.V. and Howley, P.M. (1990) Papillomaviruses. In: *Fields Virology,* second edition. (Fields, B.N. and Knipe, D.M. eds) New York: Raven Press, pp1651-1676.

Shibata, D., Fu, Y.S., Gupta, J.W., Shah, K.V., Arnheim, N. and Martin, W.J. (1988) Detection of HPV in normal and dysplastic tissue by the PCR. *Laboratory Investigation,* **59**, 555-559.

Shope, R.E. (1933) Infectious papillomatosis of rabbits. *Journal of Experimental Medicine*, **58**, 607-624.

Shope, R.E., Mangold, R., McNamara, L.G. and Dumbell, K.R. (1958) An infectious cutaneous fibroma of the Virginian white-tailed deer (*Odocoileus virginianus*). *Journal of Experimental Medicine,* **108**, 797-802.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology,* **93**, 503-517.

Stanley, M. (1990) Genital papillomaviruses, polymerase chain reaction and cervical cancer. *Genitourinary Medicine,* **66**, 415-417.

Steele, J.C. and Gallimore, P.H. (1990) Humoral assays of human sera to disrupted and non-disrupted epitopes of human papillomavirus type 1. *Virology,* **174**, 388-398.

Steger, G., Olszewesky, M., Stockfleth, E. and Pfister, H. (1990) Prevalence of antibodies to human papillomavirus type 8 in human sera. *Journal of Virology,* **64**, 4399-4406.

Steinberg, B.M. and Abramson, A.L. (1985) Laryngeal papillomas. *Clinics in Dermatology,* **3**, 130-138.

Sterling, J., Stanley, M., Gatward, G. and Minson, T. (1990) Production of human papillomavirus type 16 virions in a keratinocyte cell line, *Journal of Virology,* **64**, 6305-6307.

Strafuss, A.C., Smith, J.E., Dennis, S.M. and Anthony, H.D. (1973) Sarcoid in horses. *Veterinary Medicine and Small Animal Clinician*, **68**, 1246-1247.

Strike, D.G., Bonnez, W., Rose, R.C. and Reichmann, R.C. (1989) Expression in *Escherichia coli* of seven DNA fragments comprising the complete L1 and L2 open reading frames of human papillomavirus type 6b and localisation of "common antigen" region. *Journal of General Virology,* **70**, 543-555.

Sundberg, J.P., Burnstein, T., Page, E.H., Kirkham, W.W. and Robinson, F.R. (1977) Neoplasms of equidae. *Journal of the American Veterinary Medical Association,* **170**, 150-152.

Svendsen, E.D., ed. (1989) *The Professional Handbook of the Donkey.* Sidmouth: Sovereign Printing Group.

Tarwid, J.N., Fretz, P.B. and Clark, E.G. (1985) Equine Sarcoids: a study with emphasis on pathologic diagnosis. *Compendium on Continuing Education for the Practising Veterinarian*, 7, S293-S300.

Teifke, J.P. and Weiss, E. (1991) Detection of bovine papillomavirus DNA in equine sarcoids using the polymerase chain reaction. *Berliner und Muenchener Tieraerztliche Wochenschrift,* **104,** 185-187.

Thackray, A.C. (1978) The male reproductive organs, malignant epithelial tumours. In: *Systemic Pathology,* vol. 4, second edition. (Symmers, W. St. C. ed.) Edinburgh: Livingston, pp1555-1556.

Thomsett, L.R. (1979) Differential diagnosis of skin diseases in the horse. *Equine Veterinary Journal,* 2, 46-51.

Tidy, J.A., Vousden, K.H. and Farrell, P.J. (1989) Relation between infection with a subtype of HPV 16 and cervical neoplasia, *Lancet*, **i**, 1225-1227.

Torre, G.D., Muttini, M., Ballinari, D., Ferrari, G., Pierotti, M.A. and Porta, G.D. (1988) Characterisation of papillomavirus polypeptides from bovine cutaneous fibropapillomas. *Journal of General Virology,* **69**, 2919-2924.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the* USA, **76**, 4350-4354.

Trenfield, K., Spradbrow, P.B. and Vanselow, B. (1985) Sequences of papillomavirus DNA in equine sarcoids. *Equine Veterinary Journal,* **17**, 449-452.

Turrel, J.M., Stover, S.M., and Gyorgyfalvy, J. (1985) Iridium-192 interstitial brachytherapy of equine sarcoid. *Veterinary Radiology,* **26**, 20-24.

Valle, S.-L. (1987) Dermatological findings related to human immunodeficiency virus infection in high risk individuals. *Journal of the American Academy of*

Vanselow, B.A., Abetz, I., and Jackson, A.R. (1988) BCG immulsion therapy of equine sarcoid. *Equine Veterinary Journal,* **20**, 444-447.

Voss, J.L. (1969) Transmission of equine sarcoid. *American Journal of Veterinary Research,* **30**, 183-191.

Watson, R.E., England, J.J. and Larson, K.E. (1972) Cultural characteristics of a cell line derived from an equine sarcoid. *Applied Microbiology,* **24**, 727-731.

Watson, R.E. and Larson, K.A. (1974) Detection of tumour-specific antigens in an equine sarcoid cell line. *Infection and Immunity*, **9**, 714-718.

Webster, C.J. and Webster, J.M. (1985) Treatment of equine saroids with BCG. *Veterinary Record*, **116**, 131-132.

Wheat, J.D. (1964) Therapy for equine sarcoids. *Modern Veterinary Practice*, **45**, 62.

Williams, I.F., Heaton, A. and McCullagh, K.G. (1982) Connective tissue composition of the equine sarcoid. *Equine Veterinary Journal,* **14**, 305-310.

Williams, J.F. (1989) Optimization strategies for the polymerase chain reaction. *Biotechniques*, **7**, 762-767.

Wilson, D.G. Peyton, L.C. and Wolf, G. (1987) Immediate split-thickness autogenous skin grafts in the horse. Case reports on the treatment of equine sarcoids in 3 horses. *Veterinary Surgery*, **16**, 167-171.

Wikstrom, A., Lidbrink, P., Johansson, M. and von Krogh, G. (1991) Penile human papillomavirus carriage among men attending Swedish STD clinics. *International Journal of Sexually Transmitted Diseases and AIDS.***2**, 105-109.

Winston, T., Rings, M. and Wyman, M. (1979) Treatment of equine sarcoids. Letter, *Journal of the American Veterinary Medical Association,* **175**, 775.

Wood, A.L. and Spradbrow, P.B. (1985) Transformation of cultured equine fibroblasts with a bovine papillomavirus. *Research in Veterinary Science,* **38**,

Wright, P.A. and Wynford Thomas, D. (1990) The polymerase chain reaction: miracle or mirage? A critical review of its uses and limitations in diagnostic research. *Journal of Pathology*, **162**, 99-117.

Wyman, M., Rings, M.D., Tarr, M.J. and Alden, C.L. (1977) Immunotherapy in equine sarcoid: a report of two cases. *Journal of the American Veterinary Medical Association,* **171**, 449-451.

Wyn-Jones, G. (1979) Treatment of periocular tumours of horses using radioactive gold-198 grains. *Equine Veterinary Journal,* **11**, 3-10.

Wyn-Jones, G. (1983) Treatment of equine cutaneous neoplasia by radiotherapy using Iridium-192 linear sources. *Equine Veterinary Journal,* **15**, 361-365.

Youatt, W. (1866) The skin and its diseases; warts. In: *The Horse.* London: Longmans, Green and Company, p357.

Zeidner, N. and Bracken, F. (1985) Immunotherapy for periocular sarcoid in a mule. *Modern Veterinary Practice*, 891-894.

Zhou, J., McIndoe, A., Davies, H., Sun, X.-Y. and Crawford, L. (1991) The induction of cytotoxic T-lymphocyte precursor cells by recombinant vaccinia virus expressing human papillomavirus type 16 L1. *Virology*, **181**, 203-210.

zur Hausen, H. (1985) Genital papillomavirus infections. *Progress in Medical Virology,* **32**, 15-21.

zur Hausen, H. (1991) Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology*, **184**, 9-13.