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Viruses of the common brushtail possum

(Trichosurus vulpecula)

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Abstract

A tissue culture survey was conducted to detect viruses in possums. Up to 14 tissues from 93 wild caught possums were inoculated (co-cultivation) onto three marsupial cell lines. Possum primary cell cultivation was also developed throughout the survey period and together these procedures sought to detect viral infections as overt clinical disease, as unapparent illnesses or present in a latent form. Three passages of seven days duration were routinely performed. Haemadsorption tests (chick, guinea pig and human "O" RBCs at 37°C) and examination of stained monolayers (chamber slides) were completed for the third passage. A few adenovirus-like particles were identified by electron microscopy in one of two possums' tissue cultures in which a non-sustainable cytopathic effect was detected. No haemadsorption or abnormal chamber slide cytology was demonstrated. Adenoviruses were identified by electron microscopy in faecal or intestinal contents samples from four of the survey possums.

Wobbly possum disease (WPD), a newly described neurological disease of possums, was suggested to have a viral aetiology when filtered infectious material (clarified spleen suspension from a confirmed case of WPD passed through a 0.22 µm membrane) could transmit disease to susceptible possums following intra-peritoneal inoculation. Preliminary studies into routes of transmission of WPD used a standardised tissue suspension prepared from pooled infected liver, spleen and brain tissue. Titration of the tissue suspension *in vivo* demonstrated in excess of 10^5 possum infectious doses per ml. Clinical signs associated with neurological disease were confirmed by the presence of characteristic histological lesions and a scoring system was devised to assist diagnosis.

The tissue suspension was shown to cause disease when inoculated by the gastric, tracheal, intradermal (ID) and intraperitoneal (IP) routes. Blood and urine from infected possums were shown to be infectious when inoculated by the ID and IP routes respectively. Disease was transmitted by a suspension of blood feeding mites (*Trichosurolaelaps crassipes*) injected intradermally however the transfer of live mites from an infected possum to a non-infected recipient failed to transmit WPD.

Sentinel control possums housed adjacent to experimentally infected possums did not develop WPD but when two inoculated possums were placed in group housing with five in contact controls, all possums became infected. The absence of aerosol transmission to non-contact control possums suggests that transmission requires direct contact between possums or contact with a contaminated environment. Naturally occurring transmission was demonstrated to be feasible by the cloacal / oral and intradermal routes. Further work is required to determine the relative importance of these routes under natural conditions.

A neurological disease syndrome was investigated in a wild possum population in the Rotorua district and determined to be almost identical to WPD. Comparison of the Rotorua syndrome and WPD together with histological evidence for more widespread distribution of similar disease processes suggest that WPD or variants may already be distributed throughout New Zealand. As such, WPD may be a newly recognised disease rather than a newly emergent disease.

Papillomavirus particles were detected in association with wart-like papillomas on the tail of a possum. A papillomavirus specific product was amplified by PCR and manually sequenced. Sequence comparisons and phylogenetic analysis determined a new papillomavirus type (possum papillomavirus). The survey methodology and the possum viruses described in this thesis are discussed in terms of identifying suitable viruses for use as biological control agents.

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Abbreviations

AGID	Agar gel immuno-diffusion
ATCC	American type culture collection
ATV	Antibiotic / trypsin / versene
BD	Borna disease
BLAST	Basic local alignment search tool
BPK	Baby possum kidney
BPV	Bovine papillomavirus
CAM	Chorio-allantoic membrane
CC	Co-cultivation
CC-I	Co-cultivation I
CC-II	Co-cultivation II
cDNA	Copy DNA
CMV	Cytomegalovirus
CNS	Central nervous system
CPE	Cytopathic effect
CRPV	Cotton-tail rabbit papillomavirus
DDBJ	DNA database of Japan
dNTPs	Dinucleotide triphosphates (dATP / dCTP / dGTP / dTTP)
dd NTPs	Dideoxy nucleotide triphosphates (ddATP / ddCTP / ddGTP / ddTTP)
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulphoxide
EBV	Epstein Barr virus
EGTA	Ethylene glycol-bis (β -aminoethyl ether) N, N, N', N'-tetraacetic acid
EHV-1	Equine herpesvirus-1
ELH	Earles Lactalbumin hydrolysate
ELISA	Enzyme linked immunosorbant assay
EM	Electron microscopy
EMBL	Database run by European Bio-informatics Institute
EPP	Explant primary passage
EVPV	Epidermodysplasia verruciformis papillomavirus
FBS	Foetal bovine serum
FCM	Filtered conditioned medium
GenBank	Database (National Center for Bio-technology Information)
GIT	Gastrointestinal tract.
GM	Growth medium
H & E	Haematoxylin and eosin
HI	Haemagglutination inhibition
HPV	Human papillomavirus
IC	Intra-cerebral
IEM	Immune electron microscopy

IFAT	Immunofluorescent antibody test
IP	Intraperitoneal
MaHV	Macropodid herpesvirus
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MEM	Minimum essential medium
MM	Maintenance medium
MVE	Murray valley encephalitis
NCBI	National Center for Bio-technology Information (GenBank)
OPK	Opossum kidney
ORF	Open reading frame
PBL	Peripheral blood leukocytes
PAUP	Phylogenetic analysis using parsimony
PCEF	Primary chicken embryo fibroblast
PDB	Protein databank (Brookhaven)
PI	Post inoculation
PID	Possum infectious dose
PIR	Protein information resource (National Medical Research Foundation)
PM	Primary medium
PPK	Primary possum kidney
PPV	Possum papillomavirus
PRT	Possum reproductive tract
PSK	Penicillin streptomycin kanamycin
PTK2	Potoroo kidney
PV	Papillomavirus
PWHV	Parma wallaby herpesvirus
RBC	Red blood cell
RE	Restriction enzyme
RF	Restriction fragment
RT	Reverse transcriptase
SAPU	Small animal production unit
SNT	Serum neutralisation test
TCID	Tissue culture infective dose
TPB	Tryptose phosphate broth
WBC	White blood cell
WV	Whataroa virus

Chapter 1

INTRODUCTION

1.1 Highly adaptable and successful immigrant - beginnings.

During the mid 1800s the Australian brushtail possum (*Trichosurus vulpecula*) was introduced into New Zealand from Tasmania and eastern mainland Australia to establish a fur industry (Pracy, 1974). Numerous importations were made and relocation of brushtail possums from areas of successful liberation, with or without the support of local bodies and acclimatization societies, contributed significantly to their establishment. The Protection of Certain Animals Act, 1865 prohibited importation of several undesirable predators (foxes, venomous reptiles and birds of prey) but active lobby groups and even many scientists of the day failed to predict any negative impact accruing from the release of what was considered to be a commercially valuable folivore.

The Australian brushtail possum (hereafter referred to simply as possum) occupies a wide range of ecological niches in New Zealand, from low alpine scrub and tussock to the coast, and continues to expand into previously unpopulated northern areas (Little and Cowan, 1992). The estimated possum population in New Zealand is between 50 and 70 million (Cowan, 1991). The possum is one of the most successfully established introduced mammals and this has been attributed to the availability of forest, scrub and farmland habitats, the associated variety of foraging and sheltering opportunities, the lack of natural predators and an overall gradual decline (relative to availability) in the demand for natural fur. Evidence for the adaptive nature of possums and their wide distribution in Australia together with the mechanisms of successful dispersion in New Zealand have been summarised (Green, 1984; Jackson, 1995). The legacy for New Zealand is a tenacious pest species with far from benign economic impacts requiring ongoing time and money to control. Possums have been eliminated from some offshore islands for conservation reasons. However, prospects for eradication from the mainland are not yet realistic.

1.2 Possums damage to flora and fauna.

A huge number of observations, anecdotal and scientifically validated, document the harm done to the New Zealand forest ecosystem. The references cited in this section are intended to give an overview of the scale of the problem rather than record exhaustively the detail of numerous studies. Cowan (1990, 1991) has summarised reports of the damage caused by possums up to the present decade and provided an introduction to other relevant sources.

Studies of the ecological damage by possums warn of continuing forest and native habitat degradation. For instance, possum browsing was shown to significantly suppress fruit production by hinau trees (*Elaeocarpus dentatus*) in the Orongorongo valley (Cowan and Waddington, 1990) which had serious consequences for native bird populations and forest species. The biomass of animals and birds in the same forest was recorded and compared with several other overseas and New Zealand forests including Kapiti Island where possum elimination had begun (Brockie and Moeed, 1981). It was concluded that the biomass of possums could not be supported without forest structure transformation. Green (1984) summarised evidence from several sources that notable changes in forest structure had already occurred in the Orongorongo ecosystem. The composition of possum diets had changed significantly over the period reviewed, with recorded changes reflecting increased rarity of favoured palatable species. Dieback in the canopies of two types of Westland forest has also been documented and correlated with the abundance, distribution and feeding preferences of possums (Rose *et al.*, 1992; Rose *et al.*, 1993).

Introduced predators and vermin pose the most serious threat to native birds in direct terms but possums make a significant contribution by competing for food resources (Fitzgerald, 1984; Cowan, 1991) and by plundering nests. Scientific findings have been widely reported by the press and special interest groups in non-refereed articles. There is a culture of anti-possum awareness that has stemmed from this documented damage coupled with a judicious amount of emotive presentation. "Small, cute and public enemy no. 1" (anon, 1991), "Possum war in the deep south" (Neal, 1991), "Possum: an ecological nightmare" (Seitzer, 1992).

1.3 Possums as a wildlife reservoir of *Mycobacterium bovis*.

In a study to investigate the susceptibility of marsupials to bacteria pathogenic for eutherian mammals, it was found that possums were susceptible to *Mycobacterium tuberculosis* and *M. bovis* (Bolliger and Bolliger, 1948). Fatal infection was demonstrated following intra-peritoneal and intra-muscular injection of the mycobacterial cultures. Peritoneal exudate and lung tissue from inoculated possums (following death) was infectious to further possums by oral administration and a single possum in contact with infected possums developed pulmonary tuberculosis. Observations that possums were susceptible to *M. bovis* in a natural environment were confirmed in 1967 (Ekdahl *et al.*, 1970). Hypotheses from these observations for the potential of wildlife species in New Zealand to complicate the epidemiology of the disease and significantly hamper control operations were far-sighted but under-stated. The grazing behaviour of possums (Harvie, 1973; Green and Coleman, 1986) and the suppurative and fistulous nature of tuberculosis lesions in this species (Stockdale, 1975; Cooke *et al.*, 1995) also suggested a ready means of pasture contamination and transmission of infection to livestock. However, the mechanisms of transmission of *M. bovis* from possums to susceptible livestock species appear complex (Paterson *et al.*, 1995; Paterson and Morris, 1995; Sauter and Morris, 1995a; Sauter and Morris, 1995b) and more work is required to determine the relative importance of direct interactions between stock and tuberculous (moribund) possums and acquisition of infection from a contaminated environment. Maintenance of *M. bovis* infection in populations of possums is hypothesized to occur by pseudo-vertical transmission and social interactions fostered during the breeding season with indirect transmission (with the exception of contaminated den sites) considered to be relatively unimportant (Jackson, 1995; Morris and Pfeiffer, 1995).

The prevalence of infection by *M. bovis* in possums in an endemic area is thought to be frequently underestimated where such assessments have been based on gross lesions (Cooke *et al.*, 1995). Estimates of true prevalence, incorporating this suggestion and the results of computer modelling studies, indicate that between 15 and 20% of possums are infected during their lifetime, many as juveniles (Morris and Pfeiffer, 1995). That the incidence of *M. bovis* infection in livestock is causally related to the *M. bovis* infection status of adjacent possum populations can be presumed by the sharp drop in the prevalence of infection in cattle when possum control measures are instituted. This is reflected in national figures (O'Neil and Pharo, 1995) and for localised problem areas (Livingstone, 1991).

1.4 Control strategies - past and present.

Interrelated aspects of possum control with relevant regulatory and statutory obligations have been summarised in a recent report by the Parliamentary Commissioner for the Environment (Hughes, 1994) which confronts the magnitude of the possum "problem" and the very real threat to New Zealand's continuing ability to export primary produce. Control, as opposed to management of the possum population and prevalence of *M. bovis* infection in this species is a relative term used to imply a situation which has not yet been achieved.

There are two management goals. The reduction of possum numbers in general, with strategic control measures in areas where bovine tuberculosis is a special problem. The elimination of infected stock in endemic bovine tuberculosis areas by a test and slaughter policy. Simultaneous enforcement of movement restrictions on stock from infected properties and areas aims to prevent *M. bovis* from becoming established in previously uninfected wildlife populations. The expense of large scale possum control operations is borne by the Animal Health Board. Eradication of bovine tuberculosis is funded directly and indirectly by the farming industry which is also encouraged to participate in possum control (O'Neil and Pharo, 1995).

The current limited control of possums that is achieved is to a very large extent reliant on the use of sodium monofluoroacetate (1080) poison (Livingstone, 1994). New Zealand has the highest use of this poison on a global scale and, while extremely effective in the absence of significant bait shyness (Hickling, 1994), there are controversial issues regarding its continued use (Williams, 1993). Testing both offshore and within the New Zealand ecosystem has confirmed that 1080 neither remains for substantial periods in the environment nor builds up in the food chain (Eason *et al.*, 1993; King *et al.*, 1994; Walker, 1994; Parfitt *et al.*, 1994). However, poisoning of non-target domestic species and wildlife is a major concern and one that increasingly disturbs the public. A review of non-target bird casualties from 70 poisoning operations suggested that, with improvements in bait formulations and acceptance trials, relatively few species and numbers have been accidentally killed and that non-target species may benefit from the increased health of forest ecosystems to an extent that outweighs the risks of accidental poisoning (Spurr, 1994b; Morgan, 1994). Limited studies on non-target invertebrates have not revealed a threat to those species which have been investigated (Spurr, 1994a) but loss of honey bees and honey

contamination has been reported (Hughes, 1994). The propensity for conservationally 5
important kokako wattle birds (*Callaeas cinerea*) to take baits has been reviewed (Spurr, 1994b) but monitoring of this species following 1080 associated losses has failed to detect carcasses. There are no reported ill effects on native bats and amphibians but there are suggestions that secondary poisoning could affect the latter and it is acknowledged that many rarer species have not been adequately monitored.

1.5 Control strategies - future directions.

Current trends in the perceived (and real) dangers of pesticide use are likely to see limitations imposed in their deployment in the foreseeable future (Fagerstone *et al.*, 1994; Williams, 1993). Currently, there are newer alternatives to the now controversial compound 1080 (Eason *et al.*, 1994; Eason *et al.*, 1996) but safety and efficacy studies require completion for target species and ecosystems in addition to gaining public acceptance in times of changing attitudes to animal welfare (Gregory *et al.*, 1996).

In direct response to the continued expense of containment of *M. bovis* infection and the urgent need to develop new control techniques, attention has been turned towards biological control of possums and/or prevention of *M. bovis* infection in the same species by vaccination (Allison, 1992). An effective vaccination based control programme for a disease in wildlife has recently been illustrated in the control of rabies in foxes in Europe (Brouchier *et al.*, 1995). The formulation of a national strategy to address the possum population and prevalence of *M. bovis* infection in possums (Allison, 1992) outlined areas requiring scientific research including the development of humane biological control methods. The potential use of biological vectors for reproductive control strategies, immuno-contraception (Tyndale-Biscoe, 1991), are seen as more publically acceptable than agents which seek to reduce populations through disease and death (Jolly, 1993). Parallel investigations to uncover agents and strategies for possum control were commissioned and surveys to document the existing parasite burden of brushtail possums undertaken (Heath *et al.*, 1994; Atkinson and Wright, 1993).

Chapter 2

LITERATURE REVIEW

2.1 SCOPE OF LITERATURE REVIEW

Infectious and parasitic diseases of the Australian brushtail possum (*Trichosurus vulpecula*) have been previously reviewed (Presidente, 1984). The possum has been implicated as a wildlife reservoir of two noteworthy bacterial zoonoses, leptospirosis and bovine tuberculosis. The present review does not systematically examine the literature of either disease in possums. However, the cost of bovine tuberculosis to traditional farming systems as a rationale to fund research into possum control has been discussed (chapter 1) together with the impact of this highly adaptive species on native flora and fauna (Kerle, 1984; Green, 1984; Fitzgerald, 1984).

Aspects of possum evolution, physiology, behaviour, ecology and environmental impact are beyond the scope of this review but a broad introduction to the literature on possums may be found in the multi-authored text "Possums and Gliders" (Smith and Hume, 1984) and more succinctly in "The Handbook of New Zealand Mammals" (Cowan, 1990). A possum bibliography, compiled by the Forest Research Institute (Morgan and Sinclair, 1983), further lists early physiological studies as well as recording damage to the N.Z. ecosystem. Dietary requirements and aspects of possum handling and husbandry have been considered from the point of view of maintaining healthy captive animals (Presidente, 1982) but the recent increase in observational studies and experimental manipulations is such that an updated publication of current husbandry techniques would be well received.

A focused review of the literature was commissioned by the Department of Conservation and summarised the potential for recognised agents to provide biological control for the escalating possum population in New Zealand (Wilks, 1990). This database was defined

by electronic means and provided access to a comprehensive collection of references targeting diseases of possums and marsupials. One of the main findings from this review was the relative absence of reports of bacterial and viral isolations in marsupials compared to the identification of other parasites (no viruses had been reported from possums). Secondly, no agents were identified with suitable characteristics for immediate adaptation towards biological control of possums. Finally, it was suggested, there was considerable potential for a systematic search to uncover useful agents either in possums or a closely related species and that possums were unlikely to differ significantly from other vertebrates by lacking a reasonably diverse microbial flora.

The main purpose of this literature review is to record the recent interest in, and search for, viral infections of possums and other marsupials. Techniques that have been used to detect viruses in other species or may be applicable for this purpose in possums provide a second theme. Finally, the literature review focuses on papillomaviruses for the background information necessary to discuss the possum papillomavirus which was identified by the author during this study (chapter eight).

2.2 VIRUSES IN POSSUMS

2.2.1 Isolations.

The first isolation of a virus from possums occurred in 1995 when O'Keefe (pers. comm.) reported destruction of primary chicken embryo fibroblast (PCEF) monolayers following inoculation of spleen and liver from possums infected with a newly described, transmissible and fatal neurological disease "wobbly possum disease" (WPD) (Macintosh *et al.*, 1995). Preliminary data suggested that the virus has an RNA genome, a lipid envelope and a diameter of 40-60 nm with the former figure representing the capsid diameter and the latter the fully enveloped particle (O'Keefe, 1996). Using a range of preparative techniques, including iso-osmotic gradients, the size of the infectious particles was revised to approximately 60 nm (O'Keefe and Wickstrom, 1998a). Wobbly possum disease has met many criteria for having a viral aetiology (Koch's postulates have been partially fulfilled) and there is a considerable body of evidence which suggests that the tissue culture isolate is the causal agent of WPD (O'Keefe and Wickstrom, 1998a). Details of this newly discovered disease of possums and efforts to independently isolate the causative agent and establish routes of transmission are described in chapters four, five and six of this thesis.

2.2.2 Possum adenovirus.

Adenovirus precipitating antibodies were detected in the sera of possums by agar gel immunodiffusion (AGID) assay (Rice *et al.*, 1991). The antigen was appropriately prepared from canine adenovirus type-1 (CAV-1) and 231 possum sera from five locations were tested by AGID using reference antibodies prepared in mice and canine serum with antibodies to CAV-1. Eight positive reactions (3.5%) were obtained in the possum sera and these were interpreted as possum antibodies binding to group reactive, mammalian adenovirus antigens. When group reactivity is demonstrated, stronger reactions (higher titres) are generally encountered when the homologous virus is used in type-specific tests such as the serum neutralisation test (SNT). This, taken together with the overall low sensitivity of the AGID, suggested that the observed prevalence of adenovirus antibodies in possums was a conservative estimate. In contrast with the reference mouse and canine sera, possum sera reactive in the group specific AGID assay failed to neutralise CAV-1 in the SNT. These results first suggested the presence of a

possum adenovirus.

An electron microscopic survey of possum intestinal contents for viral particles (Rice and Wilks, 1996) demonstrated adenoviruses in six percent of samples lending weight to the hypothesis that possums are host to at least one member of this virus family. Attempts to propagate this adenovirus *in vitro* have to date been unsuccessful (Rice, pers. comm.). In addition, challenge of a juvenile possum with an adenovirus suspension, derived from intestinal contents, failed to establish infection (Rice, unpublished data). More recently, an adenovirus antigen detection ELISA (IDIEA™ Adenovirus; Dako Diagnostics Ltd) based on group reactive monoclonal antibodies, failed to detect the presumed possum adenovirus in samples of intestinal contents in which adenovirus-like particles had been identified by electron microscopy (EM) (Hadya, pers. comm.). However, degenerate primers for a conserved portion of the polymerase gene (E2B) of adenoviruses have been able to amplify DNA molecules of the expected length using the polymerase chain reaction (PCR) (Thomson pers. comm.). Sequencing of purified amplicons demonstrated a strong similarity to deposited adenovirus sequences and that all samples contained the same adenovirus sequence, presumed to have originated from a possum adapted strain.

2.2.3 Possum herpesvirus.

Herpesvirus-like particles were identified by EM in two of 100 samples of intestinal contents as both naked capsids and fully enveloped particles (Rice and Wilks, 1996). While this may appear an unusual site to locate an enveloped herpesvirus, there are precedents for such a finding. Macropodid herpesvirus-2 (MaHV-2) was isolated from the mesenteric lymph nodes of a dorcopsis wallaby (*Dorcopsis muelleri luctuosa*) that died with fulminating infection by MaHV-2 (Callinan and Kefford, 1981; Wilks *et al.*, 1981). Erythematous lesions in the gastro-intestinal tract and histologically apparent degenerative changes in association with inclusion body formation were described in two groups of wallabies afflicted with this condition in different years (Callinan and Kefford, 1981). Lesions were also described in the lung, liver, conjunctivae and vagina. An acute, focal, ulcerative gastritis of moderate severity was described in one parma wallaby (*Macropus parma*) experimentally inoculated with (presumed) Macropodid herpesvirus-1 (MaHV-1) (Acland, 1981).

Enveloped herpesvirus particles, believed to have originated from productive infections in pharyngeal or upper respiratory locations, have been detected in human stools (Armstrong and Gbewonyo, 1982). Equine herpesvirus-1 has been demonstrated by culture and indirect immuno-fluorescent techniques in the faeces of foals following intra-nasal inoculation (Patel *et al.*, 1982). Epithelial cells in the ileum and occasional lymphoblastoid cells in Peyer's patches showed evidence of herpesvirus replication. Ducklings hatched from eggs laid by duck plague virus (DPV) positive carrier muscovy, pekin and mallard ducks had low titres of DPV isolated from cloacal swabs (Burgess and Yuill, 1980).

The adult possum has a reported gastric pH of 3-4 (Tyndale-Biscoe, 1973). While one might expect a herpesvirus produced in the upper GIT to be inactivated as it passed through the acidic stomach there is potential for it to be protected in boluses of food. Alternatively, it may originate from productive infection in the lower GIT. Further investigations into the potential of possums to carry a species-specific herpesvirus, over and above the emphasis placed on gaining a herpesvirus isolation during the current study, are worthwhile. Of the various animal species intensively studied, most have at least one disease syndrome causally attributed to herpesvirus infection and several species are known to be the host of a number of distinct herpesviruses. Eight herpesviruses are recognised in human patients (Levy, 1997; Foreman *et al.*, 1997) and five in horses (Crabb and Studdert, 1996).

2.2.4 Coronaviruses and coronavirus-like particles.

Coronaviruses and coronavirus-like particles were the most frequently observed viral particles in the survey by EM of possum intestinal contents (Rice and Wilks, 1996). Of 100 samples examined, four contained coronaviruses and 14 contained coronavirus-like particles. These particles have yet to be associated with any disease entity of possums but coronaviruses of other species are well known to cause enteric infection (Holmes, 1990).

2.2.5 Retroviruses.

There are reports suggesting the presence of retroviruses in possums. Cultures of possum lymphocytes were stimulated by the addition of concanavalin A and recombinant human interleukin-2 (Meers, 1995; Meers *et al.*, 1998). In several cultures multinucleate giant cells were detected. Reverse transcriptase (RT) activity, particularly in the presence of manganese, was detected but no retrovirus particles were identified by limited EM examination of multinucleated cells from one of the lymphocyte cultures. Further work is needed to extend the above findings including standardisation of the RT assay for marsupial retroviruses. In contrast with the situation in the koala, where a retrovirus has been reported in association with leukaemia (Canfield *et al.*, 1987; Worley *et al.*, 1993), there are no reports of recognised leukaemic syndromes in possums suggestive of retrovirus infection.

Further evidence for the presence of retroviruses in possums has been obtained following the demonstration of RT activity in samples of possum blood at the University of Waikato. A PCR protocol and primers to demonstrate a conserved region of the *pol* gene of retroviruses indicated that many possum tissues contained retrovirus-related sequences, presumed to be endogenous (Baillie, 1995). Research emphasis was shifted to detection of retroviral RNA using RT-PCR and filtered possum serum and mononuclear cells in order to eliminate endogenous retrovirus-related sequences and demonstrate exogenous retroviruses. To date, RT-PCR products of the expected size (~ 130 bases) have been obtained and several subclones appeared to be identical, sharing 63% nucleotide identity with simian retrovirus-1. The RT-PCR products from one possum were appropriately labelled and used to confirm the identity of other possum RT-PCR amplicons in a Southern hybridisation assay. No such sequences were detected when genomic DNA prepared from the same cells was probed, providing further evidence that the retroviral RNA amplified from possum serum and mononuclear cells was from an exogenous retrovirus (Baillie and Wilkins, 1998). Further research would help confirm that retroviral genes (RNA) are being expressed in possum cells, and that endogenous retrovirus sequences have not been detected throughout this series of experiments. Evidence suggests that, at the very least, possums have evolved with and incorporated retrovirus-like sequences into their genome. Such endogenous sequences

have been identified in feline cells and these, following recombination between endogenous and exogenous retroviral sequences, give rise to the more pathogenic subgroups of FeLV (Squires, 1995; Neil *et al.*, 1991). While possums may also carry an independent exogenous retrovirus or be subject to a variety of dynamic recombination events between retrovirus and retrovirus-related sequences, there are no published reports of disease entities directly related to retrovirus infection in possums.

2.2.6 Other disease entities of possums.

A disease entity of possums characterised by severe non-suppurative encephalitis and retinitis, recognised in urban Sydney (Hartley, 1993) and elsewhere in Australia, has been recently re-examined in light of the identification of WPD in N.Z. (Hartley, pers. comm.). Extensive encephalitis and blindness would not be consistent with survival in the wild but a proportion of affected possums were kept alive by hand feeding. No lesions outside the CNS or retina were reported and the aetiology of the disease has not been determined.

President's summary of diseases encountered in captive possums includes unexpected deaths of apparently healthy possums with or without complication from minor injuries, bite wound abscesses or identified infectious diseases (Presidente, 1982). Stress-related mortality, as these deaths were interpreted, was briefly described as an entity in which overcrowding and social factors were considered to play a part. Affected possums became depressed and inappetent, often developing a watery diarrhoea prior to death. Increased cortisol levels in affected males during this period prompted a comparison with aspects of the annual die-off of male brown antechinus (*Antechinus stuartii*), considered to be causally associated with stress-induced immunosuppression and recrudescence of cytomegalovirus infection (Barker *et al.*, 1978). Similar clinical signs have been anecdotally reported from a number of institutions in New Zealand that maintain possums for research purposes and diarrhoea appear to be a consistent finding (Hutton, 1979; Keber, 1979; Wickstrom and Cowan, pers. comm.). A syndrome characterised by depression, diarrhoea and death has caused ongoing problems for workers maintaining possums for research purposes at Landcare Research, Lincoln. Recently the condition has been described as an acute transmissible enteritis and a cytopathic agent isolated on

primary possum kidney cells (O'Keefe and Wickstrom, 1998a). Other pathogenic micro-organisms have been associated with the disease entity and further research is required to characterise the cytopathic agent and determine its role in the pathogenesis of the disease.

It has been suggested that immuno-suppression, following the stress of capture and containment, may have predisposed possums to opportunist or activated latent infections. Buddle *et al.*, (1992) demonstrated reduced reactivity of possum lymphocytes during adjustment of possums to captivity. It was concluded that a period of three to five weeks of captivity was required before "immuno-competence" for experimental purposes could be assumed. Earlier literature has hypothesised a relative deficiency in possum and opossum cell-mediated immune responses which may be relevant to capture stress but extension and substantiation of these initial studies is required (Moriarty, 1973; Moriarty and Thomas, 1983; Brožek *et al.*, 1992).

Latent infections may recrudesce under conditions of reduced immune competence and it appears that captivity associated stress makes possums susceptible, especially during an adjustment period, to a variety of exogenous opportunist pathogens. Because apparently healthy possums die unexpectedly in captivity, these circumstances should be recognised as presenting an opportunity to identify possum specific viruses.

2.3 VIRUSES OF MARSUPIALS IN AUSTRALIA

2.3.1 Arbovirus infections.

Potkay (1977) summarised reports of studies of Australian marsupials for evidence of arbovirus infections. He noted that no arboviruses had been isolated, although there was serological evidence of past infections. Antibodies to Group B arbovirus were identified in humans, macropods and a variety of other marsupial species including *Trichosurus*, *Isoodon*, *Perameles*, *Thylogale* and *Wallabia* in Queensland (Doherty *et al.*, 1964; Doherty, 1967a; Doherty, 1967b) and Victoria (French, 1967). Two out of three possums in French's study had antibodies to Murray Valley encephalitis (MVE) virus detected by complement fixation. Two out of four *Macropus parryi* and nine out of 26 (35%) *Macropus rufogriseus* were positive for Edge Hill virus by HI (Doherty, 1967b). Fourteen out of 68 (20%) *Macropus giganteus* were positive for Kokobera virus by HI (Doherty, 1967a) but sero-positivity was detected far less often in other species.

Antibodies to nine ungrouped arboviruses were detected by HI or serum neutralisation in bandicoots, kangaroos and wallabies (Doherty *et al.*, 1970). Sero-positivity was less than 20% of tested individuals (kangaroos and wallabies respectively) in all but the following cases : Mattuppa virus 16/74 (22%) and 21/70 (30%); Trubanaman virus 21/53 (40%) and 34/70 (49%); Koanyama virus 8/15 (53%) and 25/68 (37%).

A possible role for macropods in maintenance and amplification of MVE virus was investigated (Kay *et al.*, 1985) together with other domestic and wild animal species. All 14 grey kangaroos (*Macropus giganteus*) inoculated with MVE virus, including those with pre-existing antibodies, developed a viraemia which was variable in titre and duration. Six kangaroos developed titres sufficient to infect 40-50% of *Culex annulirostris* feeding from them for several days. In contrast, agile wallabies (*Macropus agilis*) did not become detectably viraemic by suckling mouse assay but a very low proportion (2%) of *Culex annulirostris* feeding on some wallabies became infected. It was concluded that grey kangaroos, second to rabbits, could play a significant role as amplifying hosts for MVE. The majority of an unspecified but small number of possum and fox sera were reported to have complement fixing antibodies to MVE following an epidemic in 1951 (French, 1973). It appears that the ability of the brushtail possum to participate in the ecology of MVE has not been thoroughly investigated.

2.3.2 Encephalomyocarditis virus.

Encephalomyocarditis virus (EMCV) was isolated from a wide range of species, including two Goodfellows tree kangaroos (*Dendrolagus goodfellowi*), following an outbreak of sudden deaths at Taronga Zoo in Sydney (Reddacliff *et al.*, 1997). The fatalities due to EMCV infection were the first reported in a zoological collection outside the United States of America. Although EMCV was only isolated from one rat it was tentatively established that wild rodents within the zoo environs were the source of the fatal infections and that other Australian marsupials were probably also susceptible to EMCV when appropriately exposed under confined conditions.

2.3.3 Macropod herpesviruses.

(i) Introduction and isolation : The first isolation of a herpesvirus from marsupials was reported in 1975 when several members of a group of parma wallabies (*Macropus parma*) at Macquarie University, Sydney became ill and died with signs of conjunctivitis, inco-ordination, respiratory râles and ano-genital vesicles (Finnie *et al.*, 1976). Necropsy revealed congestion of the liver, spleen and lungs, a mucoid tracheitis and myocardial thickening. Histological examination of tissues confirmed liver congestion and identified intra-nuclear inclusion bodies in association with focal lesions containing swollen hepatocytes with marginated chromatin. A hyperplastic bronchiolar epithelium with cellular changes similar to affected hepatocytes was noted in one animal which also had a moderately severe myocarditis and mild myocardial necrosis. Nuclear pyknosis and fragmentation of germinal centres in the spleen were also noted. A cytopathic agent, recovered from a primary culture of kidney cells prepared from one moribund wallaby, caused focal, ballooning degeneration and detachment of monolayer cells with adjacent cells forming occasional syncytia and prominent intra-nuclear inclusions. A herpesvirus was identified by electron microscopy (Finnie *et al.*, 1976).

Interestingly, the wallabies had been sourced from Kawau Island in New Zealand on two occasions and while no outside introductions were described, these wallabies remained healthy for a year in Australia prior to the outbreak. The European history of the parma wallaby, now rare in mainland Australia, has been reviewed (Finnie, 1976) and it was

suggested that the New Zealand population, if the parma wallaby was the natural host, may have originated from non-immune stock. This situation, it was reasoned, could produce the high morbidity and mortality seen in the outbreak, which is uncharacteristic of herpesvirus infection of the natural host (Finnie, 1976).

The agent, then described as parma wallaby herpesvirus (PWHV), was transmitted to three parma wallabies by intravenous injection or conjunctival/nasal exposure (Acland *et al.*, 1981). All inoculated and two in-contact wallabies died or were killed when severely affected and a thorough investigation of the pathology of experimental PWHV infection undertaken. Epidermal lesions were most obvious on the eyelids and lips but the oral, anal and genital mucosae were also involved. Lesions ranged from swelling and oedema to small vesicles and ulcers. Less consistent findings were lymph node enlargement, corneal opacity, mild pulmonary oedema and miliary white foci in the liver.

The histopathological features of the epidermal lesions included ballooning degeneration of cells in the stratum spinosum, intranuclear inclusions and multi-nucleate cells. Lesions varied in severity between animals with liver lesions ranging from subacute necrotising hepatitis to sinusoidal mononuclear cell congestion. Inclusion bodies were not seen in livers and this observation was in contrast to the natural disease outbreak. It was noted that other herpesvirus diseases with a predominantly muco-cutaneous distribution, such as the human herpes simplex viruses and feline viral rhinotracheitis, are usually non-fatal. In conjunction with serological evidence that the PWHV was widespread (Webber and Whalley, 1978) it was suggested that high morbidity and mortality in the parma wallaby indicated it was unlikely to be the natural host species (Acland *et al.*, 1981).

(ii) Epidemiology of macropod herpesvirus : Neutralising antibodies to PWHV were detected in a wide range of marsupial hosts with a higher overall prevalence of antibodies in captive animals (Webber and Whalley, 1978). Positive titres were found in *Macropus parma* (Kawau Island, N.Z.), *M robustus robustus*, *M robustus erubescens*, *M robustus hybrids*, *M giganteus*, *M rufus*, *M eugenii*, *Petrogale* spp., *Thylogale* spp., *Lagorchestes conspicillatus*, *Potorous tridactylus*, *Perameles* spp., *Isodon* spp., *Antechinus* spp., *Dasyercus cristacauda* and *Tachyglossus aculeatus*. Although 242

sera were tested, many species had too few representatives for adequate statistical analysis and, while the definitive host is likely to be a macropod or close relative, the only other conclusion to be drawn is that the virus has co-evolved with a marsupial host rather than being acquired from human beings or another animal species. Parma wallaby herpesvirus grew in 19 marsupial cell lines, including possum cells (origin not specified), and in baby hamster kidney cells. A number of commonly used laboratory eutherian cell lines were non-permissive for PWHV growth, supporting the contention for co-evolution with a marsupial species (Whalley and Webber, 1979).

Members of two sub-species of grey kangaroos (N=218) from two free-ranging populations, the Eastern grey (*M. giganteus*) from greater Melbourne and the Western grey (*M. fuliginosis*) from Kangaroo Island, were tested for antibodies to PWHV (Kerr *et al.*, 1981). Significantly more of the 69% sero-positive animals were fully mature. An increase in antibody prevalence with age is consistent with other well known herpesviruses, for example herpes simplex virus (Whitley, 1996). With close to 90% sero-prevalence in older kangaroos it was suggested that herpesviruses were endemic in the populations studied. Like other herpesviruses, persistence of latent infection with intermittent recrudescence would be expected to maintain the antibody status. The authors contended that if these data represented the true field situation, outbreaks of disease could occur from exogenous sources or by reactivation of latent infection.

Finnie (1980) described reproductive failure associated with herpesvirus infection in a group of ten tammar wallabies (*Macropus eugenii*) and contrasted this non-fatal clinical illness with severe fatal disease seen in a quokka (*Setonix brachyurus*) and a potoroo (*Potorous tridactylus*). All animals in these case studies had antibodies to PWHV and the results of the wallaby serology, but not that of the quokka or potoroo, were reported in the data on captive marsupials presented by Webber and Whalley (1978). It could be speculated that reproductive failure, from which all wallabies recovered (Finnie, 1980), represents a more host adapted form of herpesvirus infection and that the high sero-positive prevalence in grey kangaroos (Kerr *et al.*, 1981), in the absence of any reported clinical disease, implicates this species as a possible natural host and reservoir of infection for related or susceptible species.

(iii) A second macropod herpesvirus : Two herpesvirus isolations from captive dorcopsis wallabies (*Dorcopsis muelleri luctuosa*) and one from a captive quokka (*Setonix brachyurus*) were reported following separate outbreaks of fatal disease (Wilks *et al.*, 1981). Disease in the wallabies followed their introduction to enclosures containing other macropods. The wallabies died within 24 hours of being identified as moribund and the quokka, under treatment for chronic conjunctivitis, also died suddenly following the appearance of oral and genital vesicles. The dorcopsis wallabies had gross lesions in the liver, lungs, gastro-intestinal tract, conjunctivae and vagina. The quokka had lesions in the lungs, gastro-intestinal tract and at muco-cutaneous sites. In affected individuals the lungs were oedematous and congested, livers showed small yellow foci up to 1 mm in diameter, intestinal tract mucosae were focally reddened and friable and associated lymph nodes enlarged and oedematous. Lesions at the muco-cutaneous junctions consisted of reddening, vesiculation and ulceration. Histologically, disseminated focal necrosis was a consistent feature with ballooning degeneration, intranuclear inclusion body formation and ulceration detected in various epidermal and mucosal locations. The history of the outbreaks and case by case descriptive pathology together with virus isolation and culture details were reported (Callinan and Kefford, 1981). The dorcopsis wallaby and quokka herpesvirus isolates, which were themselves serologically similar, were distinguished from the PWHV by two way cross neutralisation tests. A survey of macropod sera from captive collections (N=47) revealed 44% had neutralising antibodies to the dorcopsis wallaby isolate (Wilks *et al.*, 1981).

(iv) Macropodid herpesviruses : Characterisation of the herpesvirus isolates by analysis of their DNA led to the adoption of generic nomenclature in accordance with the 1981 report of the Herpesvirus Study Group for the International Committee on Taxonomy of Viruses (Johnson *et al.*, 1985). Restriction enzyme analysis and dot blot hybridisation showed the herpesvirus isolate from parma wallabies (Finnie *et al.*, 1976) to be different from the herpesviruses isolated from other marsupials thus confirming the earlier serological study (Wilks *et al.*, 1981). Buoyant density following analytical ultracentrifugation provided further evidence that the genomes of these two viruses differed with respect to their G+C content. A genomic map based on the restriction enzyme (RE) patterns of MaHV-2 (dorcopsis wallaby herpesvirus), including four

isomeric forms, was compared with a similar situation for herpes simplex virus, which placed MaHV-2 in the Group E category of the alphaherpesviridae (Johnson and Whalley, 1987). Analysis of the genome of PWHV, renamed MaHV-1, determined that it existed in two isomeric forms, placing it in group D of the alphaherpesviridae (Johnson and Whalley, 1990). There was little hybridisation between MaHV-1 and MaHV-2.

Little research on MaHV-1 or -2 has been published this decade but comparative genomic studies and identification of the major conserved gene regions of these herpesviruses have been initiated (Thomson, pers. comm.). A study in N.Z. to determine susceptibility of possums to MaHV-1 demonstrated sero-conversion without detectable disease (Horner, unpublished data). Four individually housed possums were challenged with MaHV-1 by the intra-nasal route. After 14 days, two of the intra-nasally challenged possums received further inoculations by the intra-muscular route and all animals, including an uninoculated control, were killed and necropsied seven days later. No evidence for a disease process was identified but inoculated possums were positive for anti-MaHV-1 antibodies in a neutralisation assay. There is a need for further work in this area as possums and possum cell lines have neither been challenged with MaHV-2 nor have possum sera been screened for antibodies to MaHV-2 (Wilks *et al.*, 1981).

A recent study (Smith, 1996) investigated the *in vitro* sensitivity of MaHV-2 to eight commonly used anti-herpetic compounds to determine if they had potential for the treatment of captive macropods affected with herpesvirus infections. Also with the conservation of captive macropodids in mind, investigations of MaHV-1 and -2 gene products and genomic comparisons between these and other herpesviruses have been undertaken (Thomson, pers. comm.). Recent work in this area has generated a number of interesting questions regarding the epidemiology of MaHV-1 and MaHV-2 as these isolates have been suggested to cross react significantly in neutralisation assays (Thomson, unpublished data). Continued research in this area may further elucidate the ecology of the macropodid herpesviruses.

2.3.4 Herpesvirus infections in other marsupial species.

A presumed macropodid herpesvirus was isolated and herpesvirus particles identified by EM following examination and cultivation of liver homogenates from an acute and fatal outbreak of hepatitis in brush-tailed rat kangaroos (*Bettongia penicillata*) and rufous rat kangaroos (*Aepyprymus rufescens*) (Dickson *et al.*, 1980). No introductions to the colony had been made and rufous rat kangaroos in a mixed population in an adjacent cage remained healthy. Reactivation of a latent infection of MaHV (type not determined) and sub-clinical signs in macropods caged adjacent to a non-host species, in which a fulminating herpesvirus infection occurred, was a possible cause for this outbreak. Hepatitis associated with herpesvirus infection has also been reported in black tree kangaroos (*Dendrolagus ursinus*) from a zoo collection in Germany (Schoon and Murmann, 1985).

A fatal, generalised disease in a young common wombat (*Vombatus ursinus*), in which hepatitis was a significant feature, was attributed to herpesvirus infection (Rothwell *et al.*, 1988). Acute, necrotic lesions were identified in the gastro-intestinal tract, lymphoid tissues, liver and lung. Virus isolation was unsuccessful in this case but intranuclear inclusion bodies in hepatocytes and enterocytes were observed by light microscopy and herpesvirus capsids, enveloped particles and paracrystalline arrays in degenerating hepatocytes were seen by EM. The wombat was housed with other macropod species which have previously been shown to have neutralising antibodies to MaHV-1 and although a swamp wallaby in this enclosure had pharyngeal and oral lesions, no serological studies of in-contact animals were reported.

2.3.5 Orbivirus infections : Viral chorioretinitis of kangaroos.

Necrotising retinitis, choroiditis (including optic nerve atrophy) and mild non-suppurative encephalitis were the histopathological lesions commonly associated with an epidemic of blindness in free-ranging kangaroos in Australia (Hooper, 1996). Orbivirus particles, detected in the retina of affected animals (Durham *et al.*, 1996), were subsequently confirmed by a number of collaborators to be aetiologically associated with the disease. The PCR indicated the presence of Wallal serogroup orbiviruses in the eyes and brains of affected animals and an indirect immuno-fluorescence test clearly demonstrated the association of Wallal serogroup orbivirus antigen with the retina (Kirkland *et al.*, 1996).

Animal transmission trials have further confirmed the causal association whilst investigation of archival material suggests that a previous case of blindness in a kangaroo was due to this agent (Reddacliff *et al.*, in preparation; Hooper *et al.*, in preparation).

2.3.6 Poxviruses.

There are reports of poxvirus infections in marsupials. Poxviruses associated with papillomatous lesions were commonly found in a population of quokkas (*Setonix brachyurus*) (Papadimitriou and Ashman, 1972). Molluscum contagiosum was described in an orphan red kangaroo (*Megalela rufa*) (Bagnall and Wilson, 1974). Lesions occurred on the elbow, back and lip and were confirmed by histological and EM examination to be identical to the molluscum contagiosum lesions seen in humans. The joey was separated and orphaned during a tag and release study of 170 animals in which no other evidence for poxvirus infection was observed and the source of the virus was not determined. A papillomatous growth of 2 cm in diameter was surgically removed from an eastern grey kangaroo (*Macropus giganteus*) and found to contain poxvirus particles (McKenzie *et al.*, 1979). The lesion in the grey kangaroo was associated with a significant inflammatory response. Otherwise it was histologically similar to the lesions reported from the red kangaroo. Again this was an isolated observation with contact animals free from disease.

2.3.7 Reovirus infection.

Haemagglutination inhibiting (HI) and serum neutralising antibodies to human reovirus were found in quokkas (Stanley and Leak, 1963). The prevalence of antibodies to three types of human reovirus appeared to increase with the level of contact with humans, prompting a second study in which quokka sera were sourced and tested from an inaccessible island that lacked recorded human contact (Stanley *et al.*, 1964). Greater than 50% of quokka sera tested using similar methodology had HI antibody titres to all three reovirus types and a single isolation of a type III reovirus was made from the faeces of one quokka. Antibody was also detected to one of the three reovirus types in six to 11 (40 - 73%) of 15 unspecified kangaroo sera from the earlier study and three wallaby (*Protemnodon eugenii*) sera from a total of 11 tested in the second survey had antibodies to the reovirus type III. It was concluded that reoviruses are relatively ubiquitous and

that human infections do not serve as a source of reovirus for these species. There has been no attempt to look further for reoviruses in marsupials and no incidental findings of reovirus infection have subsequently been reported.

2.3.8 Koala retrovirus.

Leukaemia of lymphoid origin and lymphosarcoma are commonly reported forms of neoplasia in the koala (*Phascolarctos cinereus*) (Canfield *et al.*, 1987). Haematological changes suggestive of leukaemia (anaemia, lymphocytosis, abnormal lymphocyte cytology) were reported in an aged female koala that was progressively losing weight (Canfield *et al.*, 1988). Electron microscopic examination of the bone marrow of this koala revealed retrovirus-like particles, adjacent to and associated with abnormal cells. Retroviruses have been detected in koalas with lymphoid neoplasia in San Diego Zoo (Worley *et al.*, 1993). Retrovirus-like particles were revealed by EM in fixed primary cell cultures of peripheral blood lymphocytes (PBL) in the majority of clinically ill koalas investigated. Retroviral DNA (representing the reverse transcriptase portion of the polymerase gene) was detected by PCR amplification of genomic DNA from fresh PBL, buffy coat cells and cultured PBL and there was a high correlation with the positive EM results (Worley *et al.*, 1993).

2.3.9 Lesions of cytomegalic virus infections in Australian marsupials.

Lesions characteristic of infections by cytomegalic viruses have been reported in the prostate of the dasyurid marsupials *Antechinus stuartii* and *Phascogale tapoatafa* have been demonstrated (Barker *et al.*, 1981). Enlarged epithelial cells with nuclei up to four times their normal diameter containing marginated chromatin and central eosinophilic inclusions were noted as was the lack of a host inflammatory response. Electron microscopic examination of prostatic tissue revealed virus-like particles both in the intra-nuclear (IN) inclusions and in the cytoplasm. Virus-like particles were circular or rhomboid and inclusive of a well defined membrane had dimensions ranging from 60 to 130 nm. Circular particles appeared to be approximately 60 to 70 nm in diameter whereas oval particles had more variable dimensions. It was suggested that disease was secondary to immunosuppression related to the mating season (Barker *et al.*, 1978) but attempts to reproduce this disease phenomenon with corticosteroid administration were inconclusive.

Lesions suggestive of cytomegalic virus infection have also been described in dusky antechinus (*Antechinus swainsonii*), bettongs (*Bettongia gaimardi*) and little pygmy possums (*Cercartetus lepidus*) (Munday and Obendorf, 1983). Lesions in the antechinus were confined to the prostate, typical of the cytomegalic changes described above, and evoked a minimal host inflammatory response. Ultra-structural analysis of inclusion bodies in prostatic tissue revealed randomly dispersed virus particles composed of an electron dense core and surrounding capsid. In the cytoplasm many particles had a second surrounding envelope. In the bettongs and little pygmy possums, the lesions were confined to the renal collecting tubules. Virus particles in bettongs were again identified within intranuclear (IN) inclusions. They showed an approximately 70 nm hexagonal or spherical capsid surrounding a large electron dense core. The morphology of the virus particles in bettongs differed significantly from those of the antechinus and were considered to be more typical of an adenovirus. No cytoplasmic particles were seen to aid identification.

2.3.10 Other viral inclusions.

Intra-nuclear inclusions, described by light microscopy, have been reported in the renal tubular epithelium of possums (Hurst *et al.*, 1943). Several transmission experiments were performed in an attempt to demonstrate an infectious agent, with or without dietary association, and explain the general trend for increasing prevalence with captivity. The experiments were inconclusive. Barker *et al.* (1981), in referring to the Hurst paper, argued that latent virus infections in marsupial species may be widespread and induced by the stress of prolonged captivity. Intranuclear inclusion bodies in the liver of a koala, typical of herpesvirus inclusions by light microscopy, were subsequently found by EM not to be of viral origin (Condrón and Forsyth, 1986). An alternative hypothesis proposed that the irregular and granular masses of electron dense material, often contained within lysosomes, were due to metabolic changes and storage of a glycogen-like substance. Lesions similar to those reported in the koala have been seen in the liver of possums in New Zealand (Cooke, 1993; Horner, pers. comm.).

2.3.11 Miscellaneous studies.

Neutralising antibodies to mucosal disease virus were detected in two of 44 Bennett's wallabies (*Macropus rufogriseus frutica*) that were tested as part of a serological study of some infectious diseases in Tasmanian wildlife (Munday, 1972). The significance of this finding was uncertain and no subsequent studies have investigated a role for marsupials in bovine viral diarrhoea / mucosal disease. Selected marsupial species, including possums, were investigated for their susceptibility to foot and mouth disease virus. Although foot and mouth disease virus was recovered from possums following inoculation by the intra-dermal route, it was considered that marsupials (and in particular macropods), would only act as hosts or vectors of foot and mouth disease under exceptional circumstances (Snowden, 1968).

2.4 VIRUSES IN AMERICAN MARSUPIALS

2.4.1 Marsupial hosts as reservoirs for viruses of other species.

Early literature indicating the role of New World marsupials in the ecology of certain viruses has been summarised (Potkay, 1977). The studies were principally undertaken to investigate the potential for wildlife species (including marsupials) to participate in the ecology of known viral diseases of humans and domestic animals as opposed to an investigation of the ecology of the viruses of wild animals *per se*. A low incidence of rabies in wild opossums (*Didelphis virginiana*) and a high resistance to experimental rabies infection led workers in the 1950s and 60s to conclude that the opossum was not an important host for rabies virus.

Opossums (*Didelphis virginiana*) were reported to be susceptible to pseudorabies (Aujeszky's disease) virus in the absence of any evidence that this disease occurred in the wild population. In Argentina, it has been determined that a related opossum (*Didelphis albiventris*) was also susceptible to experimental inoculation but non-inoculated opossums in the same enclosure did not become infected (Ambrogi, 1982). These limited studies and the lack of observations of a naturally occurring form of the disease suggest that *Didelphis* spp. are not natural hosts for Aujeszky's disease virus.

2.4.2 Arbovirus infections.

American marsupial species have been the subject of serological investigations with respect to arbovirus infections (Potkay, 1977) and a number of arbovirus isolations have been made as summarised below. Group A Mucambo, Venezuelan equine encephalitis (VEE) and Western equine encephalitis (WEE) viruses have been isolated from *Metachirus*, *Philander*, *Marmosa* and *Didelphis* opossums. Group C viruses Itaqui and Oriboca were isolated from *Philander* and *Didelphis*. Capim and Guama group viruses were isolated from *Caluromys*, *Marmosa* and *Didelphis* opossums. Phlebotomus-borne Itaporanga virus was isolated from *Caluromys* and Piry (vesiculovirus) virus was isolated from *Philander* opossums. Serological reactions (haemagglutination inhibition, serum neutralisation and complement fixation) to over 30 viruses were identified in the opossum species described above plus *Monodelphis* and the geographic origin of reactive sera ranged from the southern United States (California, Texas, Florida, Kentucky and

Illinois) to central American locations (Trinidad, Panama, West Indies and Almirante) and south as far as Brazil.

Epidemiological aspects of VEE in the equine and human population were studied in the Guajira region of Venezuela over a nine year period to identify the maintenance host of VEE during inter-epidemic periods (Ryder, 1972). Antibodies to VEE were detected in *Didelphis* spp. and three other wild species but no isolations were made. An epidemic of VEE in south Texas in 1971 was also investigated with reference to wildlife transmission cycles (Sudia *et al.*, 1975). Only one VEE virus isolation was obtained from 4,739 wild or domestic non-equine vertebrate samples and this was from an opossum (*Didelphis marsupialis*). Further studies on these samples indicated that serological activity was far greater in large domestic animals than wild birds, mammals or reptiles. The isolation of VEE from a single opossum was of uncertain significance but coupled with a low rate of sero-positivity (5/120) in this species suggested the isolation to have been a chance event. In a study to determine the susceptibility of a range of wildlife species to experimental VEE virus infection, adult raccoons and opossums were found to be relatively resistant to infection (Bowen, 1976). When infection was demonstrated, the associated viraemia was lower than the levels of virus recovered from the blood of more susceptible rodents. It was concluded that opossums were not important as amplifiers of VEE virus during epizootics. However, during an outbreak of VEE in Guatemala over a similar period, virus was isolated from various hosts and serological investigations determined that opossums (*Didelphis marsupialis*, *Philander opossum*) were frequently infected (Scherer *et al.*, 1976).

The ecology of arboviruses belonging to the California supergroup, subsequently classified as bunyaviruses, was investigated in Iowa by testing for antibodies to Trivittatus (TVT) virus and Jamestown Canyon (JC) virus. Out of 25 opossum (*Didelphis marsupialis*) sera tested, one neutralised JC virus and three neutralised TVT virus (Pinger *et al.*, 1975). It cannot be concluded from these data that the opossum plays a significant role in the spread of TVT or JC viruses. Another study of JC virus, seeking to determine arthropod vectors, provided inconclusive evidence of the involvement of opossums (*Didelphis virginiana*) in transmission cycles with four from

25 samples seropositive by a neutralisation assay (Clark *et al.*, 1986).

Evidence that the opossum (*Didelphis marsupialis*) has a possible role in bunyavirus ecology was demonstrated when neutralising antibodies to Arboledas virus (phlebotomus fever serogroup) were detected in 28% of opossum sera tested (Tesh *et al.*, 1986). Infection of experimental opossums (*Didelphis virginiana*) with Arboledas virus resulted in viraemia and sand flies (*Lutzomyia gomezi*) fed on a viraemic possum also became infected. Transovarial transmission of Arboledas virus was subsequently demonstrated in female sand flies experimentally inoculated with virus (Tesh *et al.*, 1986). These authors proposed that maintenance of Arboledas virus in nature was complex and occurred by vertical (transovarial) transmission and an alternating marsupial-sandfly cycle.

Araguari virus, isolated from the organs of a *Philander opossum* in Brazil during early studies of arbovirus ecology, was recently investigated as part of a study to classify uncharacterized viruses (Zeller *et al.*, 1989). Morphologically this virus belongs to the *Arenaviridae*. Serological evidence indicates that HI antibodies to this virus are only high in marsupials and that the virus does not appear to be widespread.

2.4.3 Picornavirus infections.

Encephalomyocarditis virus was isolated from a dead opossum (*Didelphis marsupialis*) at a zoological park in which individuals from several species died or were affected in an outbreak (Wells *et al.*, 1989). The opossum was considered to have been an accidental host rather than have had a primary role in the outbreak.

2.4.4 Opossum adenovirus.

Isolation of an adenovirus from an opossum (*Didelphis marsupialis*) in Venezuela was reported following degeneration of primary opossum kidney cell cultures (Morales-Ayala *et al.*, 1964). The infectious agent produced intranuclear inclusions considered typical of a cytomegalovirus but subsequently these were shown to be due to an adenovirus on the basis of ultra-structure, chloroform resistance and complement fixation test reactivity with positive human sera.

2.5 APPROACHES TO THE DETECTION OF VIRAL PATHOGENS

2.5.1 Introduction.

In contrast to the many reports of serological surveys, reports of prospective tissue culture surveys of healthy wild animals for viruses have not been found in the published literature. Where these investigations have been performed and negative results obtained, circulation of the results may be limited (for example Horner, 1992). When a positive result is gained from a systematic approach, descriptors of published results may emphasise the pertinent findings and not the methodology of the survey.

Many studies and surveys have been performed in which tissue culture methods have sought to isolate viruses associated with certain disease entities or anatomical sites considered important in viral persistence or latency. For example, early research into feline viruses surveyed the viral flora of the upper respiratory tract of normal and clinically ill domestic and cattery cats (Walton and Gillespie, 1970). The isolation system was based solely on examination of primary cell cultures for CPE and cytological evaluation of stained coverslip cultures. Three passages of five days duration were performed and sampling methods were modified after initial negative results.

In order to study the epidemiology of human influenza viruses, wild birds were widely surveyed to detect the carriage rate of ortho- and paramyxoviruses once their potential reservoir status for human influenza viruses was elucidated (Ottis and Bachmann, 1983; Abenes *et al.*, 1982). Search protocols were designed to favour detection of those pathogens. Further examples of targeted search protocols in North Western Australia, where arboviruses were expected, included inoculations of pooled mosquito extracts onto vero cells, the chorio-allantoic membrane of chicken embryos and intra-cerebrally into day old mice as part of a standard isolation protocol to investigate arbovirus ecology (Wright *et al.*, 1981). In a local study to investigate the role of viral pathogens in respiratory disease of New Zealand horses, three cell lines were inoculated with nasal/pharyngeal swabs and peripheral blood mononuclear cells (Dunowska *et al.*, 1996). Isolation strategies to cultivate viruses implicated in 'poor performance' syndromes were formulated against a specified background of expected findings.

An electronic search of thesis abstracts located a very few studies that used a tissue culture protocol for survey work. None were more directly applicable than the feline example above and no protocols searched 'blindly' for pathogens in a wild population. The lack of research protocols with a similar aim to the proposed possum virus survey suggested that specific target virus(es) were being sought when viral aetiologies were suspected in clinically ill populations and applied tissue culture techniques were designed to maximise the isolation the pathogen(s).

Protocols for virus isolation, where there has been evidence for a viral disease, have been many and varied. Disease signs often suggested the type of viral pathogen involved which in turn directed the research method. In the following part of the literature review, examples of the various approaches taken to identify new viruses are described.

2.5.2 Embryonated eggs.

The chicken embryo preceded tissue culture monolayers as a sterile source of cells for virus propagation and chicken embryo manipulations have been thoroughly described (Burleson *et al.*, 1992; Versteeg, 1985). Eggs provide several routes for inoculation, a range of foetal cells and cultural surfaces, rapid differentiation of embryonic tissues and many opportunities for viruses to replicate in specialised or preferred conditions (Versteeg, 1985). The chicken embryo has not been completely superseded for primary isolation or propagation of viruses and several avian and some mammalian viruses (such as influenza viruses) are preferentially grown in live eggs or by related *in vivo* techniques. The isolation of duck plague virus, for example, utilises day old duck or 8-10 day duck embryos as a more sensitive system than avian embryo fibroblasts (Richter and Horzinek, 1993). Virulent birnavirus (infectious bursal disease), as opposed to strains of low pathogenicity, requires isolation in eggs (McFerran, 1993). Avian coronaviruses (infectious bronchitis) have been grown from tracheal explants but the main method of isolation remains inoculation into eggs (McMartin, 1993). Specialised *in vivo* isolation techniques have also been documented for orthomyxoviruses (especially influenza A viruses) and paramyxoviruses (Newcastle's disease)(Alexander, 1993; Kouwenhoven, 1993), Avian encephalomyelitis virus (Calnek, 1993) and avian poxvirus (Tripathy, 1993).

Although cell culture techniques have largely replaced *in vivo* assays for human and animal viruses, space constraints, convenience and costs (both financial and ethical), as opposed to a lack of effectiveness of previous systems, have been important factors in the change toward *in vitro* diagnostics wherever possible. There are still several instances in which eggs are a highly recommended adjunct to tissue culture techniques. Certain strains of mammalian influenza viruses are more readily isolated in eggs than in monolayer cultures of standard cell lines (McIntosh, 1990).

2.5.3 Infant mouse inoculation.

Inoculation of virus containing samples into suckling mice and other rodents was a standard method of virus isolation prior to the advent of modern cell culture techniques (Versteeg, 1985b). The requirement for these practices as part of diagnostic virology has largely past but inoculation of new-born mice is still the most sensitive isolation system for some coxsackieviruses, rhabdoviruses and arboviruses (Hsiung, 1982b). There remains a small requirement for these *in vivo* manipulations in applied virology research. Quite recently, a lyssavirus was isolated from an Australian fruit bat (*Pteropus alecto*) following the inoculation of tissue homogenates from a young fruit bat, affected with a rabies-like disease, into mice (Fraser *et al.*, 1996).

2.5.4 Isolation of viruses from cell culture systems.

(i) Virus cultivation : Potent and relatively non-toxic antimicrobials together with definition of the nutrient requirements of animal cells grown *in vitro* led to a significant increase in the use of tissue culture techniques from the 1950s. Not only were stable cell lines readily available to grow viruses but the procedures; tissue disruption, enzymatic disaggregation and fragment explantation, leading to their establishment enabled adventitious viruses from many different species an opportunity to replicate *in vitro*.

Much of diagnostic virology is based on the use of permissive cell lines. Stable cell lines from a huge variety of mammals, birds and invertebrates, available through catalogues from cryo-preserved stocks, maintain cell morphology and genetic constitution almost indefinitely with subculture. Tissues samples, exudates, swabs, sera or other material suspected of carrying a viral pathogen are inoculated (following adequate treatment to

suppress any microbial contaminants) onto a range of cells in culture such that at least one cell line is expected to support the growth of any suspected virus(es). This process is aided when a restricted subset of viruses are likely and the corresponding subset of inoculations can be performed (Hsiung, 1982a; McIntosh 1990).

Viral replication, at it's most obvious, manifests itself as a cytopathic effect (CPE) in which the phenotypic appearance of the culture differs visually from control cultures. Plaques are probably the easiest disruption of a cell monolayer to observe, but more subtle changes such as ballooning degeneration, vacuolation and giant cell formation may or may not lead to total monolayer disintegration. A variety of phenotypic changes that do not necessarily kill cells but are none the less viral-induced may need special methods for detection. For example, the production of inclusion bodies in the nucleus or cytoplasm may be detected when cultures are stained and examined cytologically (Malherbe and Strickland-Cholmley, 1980; Versteeg, 1985a; Hsiung, 1982b).

(ii) Co-cultivation : A more specialised method of virus isolation that involves the relatively careful handling of tissues and diagnostic samples containing cells in order that a proportion of the harvested cells remain in living contact with indicator cells is termed co-cultivation. This technique detects viruses that are closely cell associated, as opposed to being released and infectious in host secretions, and those that are 'latent' in the infected tissues. For example, nerve ganglia containing alpha-herpesvirus DNA, as a non-replicating episomal passenger virus, produce an active herpesvirus infection *in vitro* by co-cultivation when released from host suppression mechanisms. The same tissue inoculated as homogenised and disrupted cell debris will not result in a productive herpesvirus infection. In this context latent virus has been defined as "that virus which is detected after incubation of intact ganglionic tissue with suitable susceptible cells and not by inoculation of the susceptible cells with macerated ganglia" (Roizman and Sears, 1990).

Lentiviruses of veterinary importance have been isolated using co-cultivation techniques often in conjunction with primary cells. Caprine encephalitis virus can be isolated by the co-cultivation of buffy coat cells on goat synovial membrane cultures (Narayan and Cork,

1990). Cultivation of retroviruses is often achieved using lymphocyte cultivation systems (see below).

(iii) Stimulated lymphoblast cultures : The use of lymphocytes as an indicator cell for the presence of viruses which preferentially replicate in mononuclear cells is a form of co-cultivation. Lymphocyte culture systems, maintained *in vitro* by the addition of mitogens, have been described in the investigation of the koala retrovirus and a possible possum retrovirus (Worley *et al.*, 1993; Meers *et al.*, 1998). Feline immunodeficiency virus (FIV), was isolated following recognition of a transmissible AIDs-like illness in a Californian cattery and shortly after from a persistently leucopenic cat in the U.K. (Pederson *et al.*, 1987; Harbour *et al.*, 1988). In order to detect the virus, buffy coat cells from FIV inoculated cats were co-cultivated with mitogen-stimulated mononuclear cells from the peripheral blood of specific pathogen free cats. Feline T-lymphotrophic virus, as it was first named, was demonstrated by CPE in lymphocyte cultures, reverse transcriptase activity in culture supernatants and by the EM appearance of budding lentivirus particles.

(iv) Primary cell culture : When tissue culture methods were still relatively new, many viruses, from a wide range of species, were identified by investigation of the phenomenon of primary cell degeneration. For example, adenoviruses were first recognised in 1953 by Rowe and colleagues, during attempts to establish oropharyngeal cell lines, when a transmissible agent was observed to cause degeneration of primary cultures of tonsils and adenoids surgically removed from children (Horwitz, 1990a). Bovine adenoviruses have been isolated from primary testicle cell cultures derived from clinically normal calves (Bürki *et al.*, 1978). The term 'latent' has been applied to this situation and was also used, without the strict definition as applied to alpha-herpesviruses (above), to describe a study of virus infections in apparently healthy laboratory animals (Hsiung and Kaplow, 1969). Kidney tissue was used to establish primary cultures which were observed for viral CPE. Guinea pig herpesviruses were isolated following spontaneous degeneration of kidney cell cultures (Hsiung and Kaplow, 1969; Hudson, 1994). During the establishment of cell lines from a green Iguana (*Iguana iguana*), a spontaneous degeneration of primary liver cell cultures was determined to be due to a

herpes-like virus (Clark and Karzon, 1972).

The success rate for isolation of viruses in primary cells set a precedent for their continued use. Primary embryo fibroblast and primary explant cultures may be prepared (theoretically) from any species (Freshney, 1994a) and techniques for the specialised culture of many differentiated cell types are increasing (Freshney, 1994b).

2.5.5 Detection of occult viral replication.

Viruses can replicate in tissue culture without producing a CPE. On these occasions an alternative means of demonstrating the presence of a virus is required.

(i) Interference : Viral interference is a phenomenon whereby an infected cell culture that shows no evidence of CPE will not support the growth of a second virus that would normally propagate unchecked. This property of viruses was classically demonstrated by rubella virus (Hsiung, 1982c) which neither showed evidence of cell destruction in vero cells nor allowed echovirus-11 to replicate. Non-cytopathic and cytopathic strains of bovine viral diarrhoea virus may behave in a similar manner but the technique has limited application as this phenomenon has been documented for very few viruses.

(ii) Haemadsorption and haemagglutination : These related techniques are more widely used to detect occult viral replication. Viral proteins, glycoproteins and other products of virus-directed cell metabolism often demonstrate haemagglutinating properties (cell culture lysates) and haemadsorbing phenomena when cell monolayers, in the absence of a CPE, are incubated with an appropriate red blood cell (RBC) species. Recognition of this phenomenon dates from 1941 when influenza virus was reported to agglutinate chicken RBC (Rosen, 1969) and since then the haemagglutinating properties of viruses have been systematically investigated and reported (Howe and Lee, 1972). Examples of haemagglutinating viruses include orthomyxoviruses (influenza viruses), paramyxoviruses, adenoviruses, reoviruses and arboviruses. Whataroa virus, a former group A arbovirus reclassified as an alphavirus, replicates in mosquito cells without a CPE, yet agglutinates goose red blood cells under strict conditions (Ross *et al.*, 1963). Haemadsorption and haemagglutination (notably haemagglutination) have also been

adapted for use as a serological test (haemagglutination inhibition) (Hierholzer and Suggs, 1969; Hierholzer *et al.*, 1969a).

2.5.6 Isolation of arboviruses.

Arboviruses multiply within and are spread between susceptible vertebrate hosts by arthropods (Monath, 1988) and isolation of these agents is to some extent specialised. These viruses may be isolated in insect tissue culture, in embryonated eggs, on primary chicken and duck embryo fibroblasts and by intra-cerebral (IC) inoculation of suckling mice (Hsuing, 1982a; McIntosh, 1990). The initial isolations of Western Equine Encephalitis (1931) and St Louis Encephalitis (1933) were made by IC inoculation of mosquito extracts into suckling mice (Olitsky and Casals, 1959; Olitsky and Clarke, 1959). Once the suckling mouse had been identified as a sensitive isolation system, a steady stream of research began to unravel the nature of the causal agents, their host range and epidemiology.

Over 500 arboviruses, now contained in many taxonomic families, have been catalogued and activity by this broad group was often first identified by serological methods in response to characteristic disease signs in human or domestic animal hosts. The historical classification of arboviruses into serogroups by haemagglutination inhibition (HI), complement fixation and IC neutralisation allowed arboviruses to be identified and grouped by cross-reactive antigens (Casals and Reeves, 1959). Isolation of an arbovirus was then pursued using combinations of protocols mentioned above. An illustration of this approach in Westland, N.Z. has been documented in which a serological survey identified a group B arbovirus, by haemagglutination inhibition, that was related to the reference arboviruses employed (Hogg *et al.*, 1963; Maguire *et al.*, 1967). Inoculation of mosquito extracts, from high sero-prevalence areas, onto chicken monolayer cultures isolated a plaque forming agent (Whataroa virus) which was lethal to suckling mice following IC inoculation (Ross *et al.*, 1963).

Murray Valley encephalitis virus (MVE) is typical of flaviviruses associated primarily with encephalitis (Monath, 1990). Experimental infections in macropods and other domestic animals were induced in order to investigate their role as maintenance or

amplifying vertebrate hosts (Kay *et al.*, 1985). Virus stocks were grown in chick embryos and suckling mouse brains, assays for duration and intensity of viraemia performed in litters of mice and serological responses of hosts investigated using HI.

In order to isolate an arbovirus *in vitro*, relatively modern insect tissue culture practices should ideally be supported by tried and successful protocols such as primary chicken and duck cell cultures and *in vivo* isolation in suckling mouse brains.

2.5.7 Latency.

The term latent has been widely used in virology in more than one context as indicated above. Baillière's veterinary dictionary (Blood and Studdert, 1988) defines latent as "dormant or concealed; not manifest; potential". The "latent period" and the "latent period of replication" specifically refer to the seemingly inactive period between exposure and manifestation of response and the time to production of progeny virus respectively. Latency in broad terms, and irrespective of the mechanisms, is a feature of many virus families that can influence the ease with which viruses can be isolated from differing virus containing samples.

Alpha-herpesvirus latency has been mentioned above as a specific case. Latency is also a characteristic of Epstein-Barr virus (EBV), a gammaherpesvirus, which persists in the majority of sero-positive hosts for their lifetime. It is unclear whether EBV achieves true latency in oro-pharyngeal locations (chronic productive infections can equally explain long term shedding) but clearance from B lymphocytes is apparently evaded through a quiescent state ('latency') whereby viral gene products (signals for immune surveillance) are not produced (Miller, 1990). Isolation of EBV can be achieved by immortalisation of donor (neonatal) lymphocytes following inoculation of saliva or co-cultivation of lymphocytes from the infected individual (Miller, 1990).

Human adenoviruses appeared to be 'latent' in pharyngeal lymphoid tissue when first demonstrated in explant cultures of surgically removed adenoids. Recovery of human adenoviruses from purified tonsillar lymphocytes was enhanced following mitogen

stimulation of the purified cultures prior to cultivation (Van der Veen and Lambriex, 1973). However, in this study adenovirus was also recovered from unpurified suspensions of tonsillar lymphocytes and while a feasible methodological explanation supported the hypothesis for adenovirus latency in lymphocytes, true latency in tonsillar tissue has been subsequently confirmed (Horwitz, 1990b).

Latency suggests a degree of viral quiescence that requires samples to be handled carefully with respect to the preservation of cell viability and often cultured for weeks in order to obtain productive infections. Primary explant and prolonged co-cultivation procedures have been associated with the successful isolation of latent and fastidious viruses and complex isolation protocols continue to find application in the investigation of viral disease entities. For example, equine adenovirus-1 was isolated from cases of cauda equina neuritis using a combination of primary explant and co-cultivation procedures (Edington *et al.*, 1984). Adenovirus CPE was first detected following four 10 - 14 day passages. Recently, Kaposi's sarcoma (KS) associated herpesvirus (KSHV), initially detected as gamma-herpesvirus related sequences in KS of AIDS patient's, was isolated following the establishment of primary cell cultures from KS tissue and co-cultivation of these cells with a human cell line (Foreman *et al.*, 1997).

2.5.8 Electron microscopy.

Viruses can be demonstrated in samples of infected tissues, secretions and excreta or infected tissues (thin sections) following appropriate preparation and heavy metal staining (Almeida and Waterson, 1969; Palmer and Martin, 1988). Most viruses are detectable by EM when particles can be concentrated above a threshold for visualisation (10^5 - 10^6 particles/ml) which varies with the type of virus (Grimley and Henson, 1983). Immune-EM (IEM) techniques aim to concentrate virus particles and effectively lower their detection threshold for diagnostic work in addition to numerous research applications (Almeida and Waterson, 1969). Electron photomicrographs illustrate typical virus morphology, communicate results of morphological research and aid in viral taxonomy.

The ease with which viruses can be demonstrated by E.M. varies widely and not all infectious agents considered to be viruses have been definitively described. Particles

tentatively ascribed as the agent of Borna disease, clinically recognised for over 100 years, have recently been demonstrated for the first time (Compans *et al.*, 1994; Zimmermann *et al.*, 1994; Sasaki, 1993). Hepatitis C virus, neither cultivated nor visualised, was taxonomically assigned to the flaviviridae on sequence data (White and Fenner, 1994b). Inability to culture and visualise a virus has also been documented in the case of big liver and spleen disease of broiler chickens (Williams *et al.*, 1993).

Electron microscopy has been used to screen wild, domestic animal and human populations for viruses. Adenovirus, herpesvirus, coronavirus and coronavirus-like particles were found when the gastro-intestinal contents of 100 possums were investigated for viruses using negative contrast EM (Rice and Wilks, 1996). Immune-EM techniques, using specific antisera for various virus families, in conjunction with EM were used to demonstrate viruses and virus-like particles associated with diarrhoeic and normal faeces of cats and dogs (Marshall *et al.*, 1984; Marshall *et al.*, 1987), to investigate a causative role for reoviruses in human infantile diarrhoea (Kapikian *et al.*, 1974) and to evaluate concentration and staining methods for these investigations (Armstrong and Gbewonyo, 1982). Immune-EM was also considered to be sufficiently sensitive for the demonstration of antigenic relatedness between isolates of San Miquel sea lion virus, vesicular exanthema of swine virus, various calicivirus isolates from marine animals and a vaccine strain of feline calicivirus (Smith *et al.*, 1978). Therefore, specific antisera may be able to increase the sensitivity of survey protocols employing EM to screen populations for certain viruses.

2.6 MOLECULAR APPROACHES TO IDENTIFICATION OF VIRUSES

2.6.1 Introduction.

The previous two decades have witnessed a vast increase in research in virology at the molecular level. New insights into viral replication, ecology, taxonomy and epidemiology have resulted from the application of numerous molecular protocols. There are many powerful molecular biological tools for the investigation of viruses and the diseases with which they are associated. Techniques that are applicable or could be appropriately adapted to search for viruses in a wildlife population are discussed below. Following the identification of a virus, techniques for isolation, where feasible, may be targeted accordingly.

2.6.2 Consensus primers - polymerase chain reaction (PCR) amplification.

Numerous applications of the PCR and related techniques for detection of specific conserved nucleic acid sequences have been described (Saiki *et al.*, 1985; Mullis and Faloona, 1987; Saiki *et al.*, 1988; Ehrlich and Sirko, 1994; Burke, 1996). Related viruses have structural and functional proteins in common and these similarities are reflected at the genome level in crucial, highly conserved areas. Degenerate oligonucleotide primers to conserved regions of viral nucleic acid sequences, correctly designed and orientated, initiate the amplification of target viral DNA or RNA in a PCR reaction.

Techniques associated with the PCR have been extensively employed to detect and amplify target genes (or parts thereof) of new viruses using carefully designed primers. For example, the *pol* gene of the retrovirus associated with Jembrana disease virus (JDV) of Indonesian cattle (*Bos javanicus*) was amplified using universal degenerate primers for a conserved region of the *pol* gene of retroviruses (Chadwick *et al.*, 1995). Lentivirus specific primers were then used to characterise the virus as an atypical new member of this genus as JDV was associated with an acute, severe, lymphoproliferative disorder.

2.6.3 Nucleic acid hybridisation.

Nucleic acid hybridisation is a variably sensitive and specific reaction that has been adapted to 'probe' for specific sequences (Viscidi and Yolken, 1987). Nucleic acid probes conjugated to an indicator system allow a positive hybridisation reaction to be visualised. Traditionally, isotopic labelling and auto-radiography have been used to visualise the signal but non-radioactive methods are increasingly employed (Kricka, 1992; Kessler, 1991). These reactions do not amplify the DNA sequence *per se* but allow the products of a relatively few hybridisations to be detected and provide a useful test of the authenticity of PCR products or results generated using other screening tests. For example, caprine and bovine B group rotaviruses, difficult to cultivate and detect in a screening ELISA, were demonstrated by the use of chemiluminescent probes in a Northern hybridisation (Gueguen, *et al.*, 1996). Probes were synthesized by reverse transcription of genomic RNA followed by PCR amplification to make and refine a cDNA copy (probe) for group specific rotaviral genes (RNA). A German survey characterised 35 strains of cell culture adapted rotaviruses entirely on hybridisation (type specific probe) and PCR primer specificity (Brüssow *et al.*, 1994).

Nucleic acid probes made from the conserved region of a gene in one species is a technique by which to identify and locate the homologous gene from a related species (zoo blots). This was demonstrated when the cytochrome *c* gene of *Saccharomyces cerevisiae* was used to detect the cytochrome *c* gene of *Aspergillus fumigatus* (Raitt *et al.*, 1994). This relatively simple method could be used for screening the appropriate tissues of wild animal populations for viral nucleic acid sequences using conserved sequences within virus families.

2.6.4 Antibody 'probe' techniques.

Defined antibodies can identify reactive antigens using the same antibody/antigen reaction which determines the antibody status of serologically tested individuals. The enzyme linked immunosorbent assay (ELISA) has been adapted as an antigen capture system (Crowther, 1995) and this is one method for screening clinical specimens or tissues for the presence of virus prior to attempted cultivation. The antigen ELISA presents capture antibodies in a solid phase and invariably requires samples in a fluid medium.

In contrast, the immunofluorescent antibody test (IFAT) provides the antibody probe in solution and is therefore applicable for tissue sections or cell monolayers as well as fresh, clinically derived material. The IFAT, in an indirect form, has been applied to smears of nasal and ocular secretions for the rapid detection of infectious bovine rhinotracheitis virus (Nettleton *et al.*, 1983). Indirect IFAT techniques detected early and late membrane antigens on the surfaces of human cytomegalovirus infected fibroblasts using human convalescent-phase sera (Middeldorp *et al.*, 1985) and IFAT techniques have been used to demonstrate non-cytopathic strains of bovine viral diarrhoea virus and coxsackie viruses (Greiser-Wilke *et al.*, 1991; Argo *et al.*, 1992; Munoz *et al.*, 1996). While these methods are not restricted to any particular virus family (Caul, 1992), there are no reported applications of these techniques for screening wild animal populations for viruses.

Convalescent or aged animal serum is a mixture of polyclonal antibody species reflecting a reactive specificity to certain infective agents (antigens) to which the animal has been exposed throughout its life. While this is a dynamic population of molecules, one strategy suggested in the search for unknown viruses of possums was to collect convalescent polyclonal possum serum, assuming it to contain antibodies to possum specific viruses, and label the antibodies for use in an IFAT protocol. Monolayers, inoculated with possum tissue extracts, that had not demonstrated a cytopathic effect or other indication for occult viral replication could be probed for viral antigens reactive with labelled possum antibodies.

2.7 PAPILLOMAVIRUSES

During the course of the studies described in this thesis, a papillomavirus was identified in a lesion on the tail of a possum. This brief review of papillomaviruses is therefore included here.

2.7.1 Introduction.

Papillomaviruses cause benign, proliferative skin lesions in a wide range of animal species and are causally associated with neoplastic changes to squamous and mucosal epithelia (Syrjänen *et al.*, 1987; zur Hausen, 1994). The veterinary significance of papillomavirus infection varies from unsightliness in the case of canine oral papillomatosis to production-limiting alimentary and urinary tract neoplasia in cattle in association with specific cofactors (Sundberg, 1987). In the long-lived human host, papillomavirus-associated neoplasia assumes major importance as a cause of carcinomas of epithelial and mucosal sites. Epidemiological studies now causally link papillomavirus infection with cervical cancer, the most prevalent neoplasm of women in developed countries (zur Hausen, 1994).

2.7.2 Historical perspectives.

Papillomavirus lesions have been recognised for many centuries in their benign manifestation as skin warts (Olsen, 1987). Approximately 100 years ago, the successful transmission of cutaneous papillomas was reported in a number of animal species using cell-free suspensions derived from naturally occurring papillomas. The canine oral papillomavirus was transmitted by McFadyean and Hobday (1898) and similarly, Cadeac (1901) and Ciuffo (1907) demonstrated this phenomenon with papillomas in horses and humans respectively (zur Hausen, 1994; Olsen, 1987).

Aspects of papillomavirus biology were investigated by Shope in the 1930s using the newly discovered cotton tail rabbit papillomavirus (CRPV) (Gross, 1970). Infectious CRPV particles were consistently produced in appropriately challenged cottontail rabbits (*Sylvilagus floridanus*) whereas papillomatous transformation without productive infection was typical in domestic rabbits (*Oryctolagus cuniculus*) (Olsen, 1987; Wettstein, 1987). Strong evidence for oncogenic potential was demonstrated by

Rous and Beard (1935) when cutaneous papillomas transplanted to the abdomen of domestic rabbits developed into squamous carcinomas and by Syverton (1952) when the progression of papillomas to carcinomas *in situ* over a period of six months or more was reported. Experimentation by a number of early contributors to the papillomavirus literature have been thoroughly reviewed (Gross, 1970; Olsen, 1987). Several studies between the 1930s and 1950s investigated the role of chemicals as co-carcinogens in the altered time scale for papilloma production and papillomavirus associated neoplasia. Turpentine, tar, 20-methylcholanthrene, 9,10-dimethyl-1,2-benzanthracene and the compound scarlet R have been shown to promote the production of papillomas or to suppress papillomas in favour of malignant progression (Wettstein, 1987).

Papillomaviruses have not been grown by standard cell culture techniques and systematic investigation of these agents has been hindered by the lack of *in vitro* propagation. In the 1960s, bovine papillomavirus-1 (BPV-1) was noticed to transform certain rodent cell lines from a density and anchorage-dependent, epithelial phenotype to a refractile, fibroblastic phenotype. The fully transformed phenotype showed spindle shaped cells, loss of contact inhibition and tumorigenicity in nude mice (Howley and Schlegel, 1987). Transformation induced by BPV-1 has been investigated as a model for elucidating the viral mechanisms involved in transformation. Thus, from an early stage papillomaviruses have been associated with benign, proliferative lesions, invasive neoplastic lesions and altered cell growth *in vitro*.

Prior to the era of rapid developments in molecular biology era (1975 - present), papillomaviruses were differentiated by host range and specificity, the morphological characteristics and site predilection of the papilloma lesion and serological differences between isolates within a species (Bathold *et al.*, 1974).

However, the techniques with which to investigate papillomavirus biology have increased dramatically in the last twenty five years. The genome of BPV-1 was cloned in bacteria in the late 1970s (Shah and Howley, 1990). Liquid phase nucleic acid hybridization provided information on genome relatedness and formed the basis of a typing system which has only recently been rendered obsolete (Roman and Fife, 1989; De Villiers,

1994). Hybridization following electrophoresis and Southern or dot blotting provided diagnostic information where type-specific probes were available or could be designed from known sequence information (Ting and Manos, 1990). Typing of papillomaviruses is now based upon comparisons of genome sequence for three genes; E6, E7 and L1. If the homology between papillomaviruses is less than 90% for each of the open reading frames (ORFs), the full length genome is defined as a new type (de Villiers, 1994). Comparisons are aided by computer alignment of sequence data using conserved regions within the genes of interest (Chan *et al.*, 1992; Chan *et al.*, 1995; Delius and Hofmann, 1994). Of particular interest, the L1 protein contains highly conserved sequences for all papillomavirus types with homology at the amino acid level between bovine, human and rabbit papillomaviruses approaching 50% (Pfister and Fuchs, 1987).

2.7.3 Animal papillomaviruses.

(i) Bovine papillomaviruses (BPV) : Bovine PVs have been the most intensively studied of the animal PVs. Six types are described and these can be grouped according to the nature and location of the lesions they induce and, to a lesser extent, on global distribution. Bovine PVs type-1 and -2 produce papillomas with a marked degree of fibrous tissue involvement and are correctly termed fibropapillomas (Olson, 1990). Reviewing earlier work, it was concluded that BPV-1 and BPV-2 cause benign fibropapillomas in young cattle, often under confined conditions, where direct transmission and spread via fomites is facilitated (Olson, 1987).

Bovine PV-3 has been classified from an investigation of cutaneous epithelial papillomas in Australia (Pfister *et al.*, 1979). These papillomas lack any significant fibrous involvement and were termed atypical for cattle in that they resembled cutaneous papillomas of other species. A similar finding was described in North America where the term atypical warts was applied (Barthold *et al.*, 1974). The atypical warts occurred in cattle of all ages and persisted for longer than typical fibropapillomas which commonly affect young cattle and regress spontaneously. The atypical warts were immunologically distinct from BPV-1 and failed to induce tumours in BPV-1 permissive rodents.

Bovine PV-4 was first identified in "frond-type" squamous papillomas from a study of upper digestive tract papillomas in Scotland. These papillomas were described as true squamous papillomas (as opposed to fibropapillomas) and contained PV particles in the nuclei of keratinizing layers. Purified PV particles from these lesions reproduced true squamous papillomas when injected into the oral mucosa of cattle (Jarrett *et al.*, 1984a). Typical alimentary papillomas on the tongue, palate and buccal mucosa of a cattle beast from Queensland, Australia have been characterised as BPV-4 (Bloch *et al.*, 1995).

Bovine PV-5 has usually been described in association with a filiform or 'rice grain' lesion (Olsen, 1990) but was recently identified in cauliflower-like warts (Bloch *et al.*, 1994). Bovine PV-6 was identified from a teat 'frond' papilloma with an atypical flat morphology (Jarrett *et al.*, 1984). Two abattoir surveys identified lesions on cattle teats as atypical filiform (no fibroma), atypical flat (no fibroma) or fibropapilloma (Meischke, 1979).

(ii) Bovine papillomavirus association with neoplasia : In discrete geographic localities (Scotland, Japan, Brazil, Colombia and Turkey), enzootic haematuria is associated with BPV-1 or -2 infection as well as bracken fern ingestion (Olsen, 1990). Bracken fern ingestion (and other co-carcinogens) has also been associated with carcinomas at various other sites (pharynx, oesophagus and forestomachs) in the digestive tract of cattle where this has been studied (Scotland, Brazil and Kenya) and further associations between papillomavirus infections and neoplasia are noted by several authors (Smith and Seawright, 1995; Campo *et al.*, 1994; Gaukroger *et al.*, 1993; Jarrett *et al.*, 1984a; Campo *et al.*, 1980). Neoplastic changes in the bladder endothelium, underlying tissues and at other alimentary sites include adenomas, fibromas, carcinomas, haemangiomas, haemangiosarcomas and endotheliomas (Olsen, 1990; Campo *et al.*, 1980; Campo *et al.*, 1992). The relative importance of the two aetiological mechanisms (BPV transformation and bracken fern mutagenesis) has been the subject of debate with recent information from a long term study suggesting that bracken fern may be more causally associated with the onset of tumorigenesis in the alimentary tract and that BPVs may be reactivated from latency (Campo *et al.*, 1992; Campo *et al.*, 1994).

The relative importance of BPV-1, and BPV-2 and bracken fern in the development of polypoid papillomas, enzootic haematuria and associated bladder neoplasia of cattle has not been investigated as rigorously as has BPV-4 and malignant progression in the upper alimentary tract. However, the disappearance of the disease when improved pasture replaces bracken fern in the diet of cattle suggests that mutagenic compounds in bracken fern may be an essential prerequisite for the malignant progression (Olsen, 1990).

Papillomavirus-like virions were demonstrated in conjunctival papillomas and plaques as well as in eyelid papillomas and cutaneous horn from cattle (Ford *et al.*, 1982). These lesions were not described as 'atypical' but have been considered as precursors of squamous cell carcinomas (SCC) in areas exposed to high levels of ultra-violet light. The association of papillomaviruses and SCC in these reports provided evidence for a possible role for papillomaviruses in epithelial transformation. However, a search for BPV DNA in bovine ocular squamous cell carcinoma (BOSCC) by hybridization techniques failed to detect recognised BPV sequences in BOSCC tissues or cell lines (Rutten *et al.*, 1992). Papilloma-like viruses present in the putative precursor lesions of BOSCC may contribute to induction of neoplastic change in association with other co-factors without being required for maintenance of the tumorigenic state.

(iii) Papillomaviruses of other domestic animals : Fibro-papillomas described in other ungulates (deer, elk, goat, sheep) are similar to one another and are clustered by phylogenetic analysis of sequence data (Chan *et al.*, 1992; Chan *et al.*, 1995). A papillomavirus has been demonstrated in sheep that produced lesions similar to bovine fibropapillomas (Gibbs *et al.*, 1975). Attempts to transmit the virus to cattle or goats were unsuccessful but fibrous tumours were induced in inoculated guinea pigs. Papillomaviruses were associated with hyperkeratotic lesions adjacent to a SCC of the vulva of a ewe and in hyperkeratotic lesions on the ears of sheep (Vanselow *et al.*, 1982; Vanselow and Spradbrow, 1983). A typical benign papilloma is described in horses (Vanselow and Spradbrow, 1996) but more interestingly, a fibrous tumour of horses (sarcoïd) has been associated with the presence of BPV DNA (Amtmann *et al.*, 1980; Bloch *et al.*, 1994; Otten *et al.*, 1993). The canine oral papillomavirus is associated with cauliflower-like warts which, while occasionally quite proliferative and extensive,

usually regress spontaneously (Fenner *et al.*, 1993b).

(iv) Papillomaviruses of other species : There are isolated reports of papillomaviruses in many species not specifically mentioned above. These include several species of deer, psittacine and passerine birds, rabbits, pigs, mice, elephants, a sperm whale, primates and a marsupial. A comprehensive review of papillomas in animals, including poikilotherms and invertebrates, together with the evidence for viral involvement is given by Sundberg (1987). A captive North American opossum (*Didelphis marsupialis*) developed cutaneous papillomas on the lower limb and paw of one front leg. Papillomas later developed on the pinna of the same side. Inoculation of material derived from these papillomas into 14 other young opossums failed to reproduce lesions. Cutaneous papillomas were present on the hands of one worker looking after the opossums raising the question of whether or not the opossum had been infected from the worker (Koller, 1972).

2.7.4 Human papillomaviruses.

Papillomaviruses cause a wide spectrum of benign proliferative and neoplastic events in the human host. More than 70 types of human papillomavirus (HPV) have been identified on the basis of sequence differences (Chan *et al.*, 1995) and sub-groupings of these correlate well with their biological behaviour. Epidemiological studies have sought to determine if causal associations exist between certain HPV types and neoplasia (for example cervical cancer) and increased research interest has been focused on those types associated with malignant progression (Schiffman, 1994). In spite of many methodological differences relating to sample selection, geographical and sociological variables and differences in the range of tests applied, the evidence to date that certain HPVs are the major causative agents for cervical cancer is overwhelming (Schiffman, 1994; zur Hausen, 1994).

Different human papillomavirus types are involved in proliferative lesions on all body surfaces and may be grouped by body system for description (Syrjänen *et al.*, 1987; zur Hausen, 1994). Papillomavirus have been implicated as the sole aetiological agent in benign lesions and in association with host genetic factors and co-carcinogens in those

lesions which progress to malignancy. The primary literature in this fast moving field is beyond the scope of this overview but three comprehensive review publications on human papillomaviruses are available (zur Hausen 1994, Roman and Fyfe 1989, Syrjänen *et al.*, 1987).

2.7.5 Papillomaviruses of non-human primates.

It has recently been demonstrated that several PV types are associated with genital samples from rhesus monkeys and long-tailed macaques (Chan *et al.*, 1997). Furthermore, a genital PV and a PV from the epidermodysplasia verruciformis (EV) supergroup in an Abyssinian colobus monkey were distinct from but grouped with the human genital PVs (supergroup A) and human EV PVs (supergroup B) respectively (Chan *et al.*, 1997a). One implication is that papillomavirus evolution (tropism within host diversity) predated the evolution of monkeys and apes. It is currently unknown whether or not a similar range of PVs exists in other animal species or whether the range of PVs seen in primates reflects a bias in systematic research. With the exception of cattle, each of the many species reported to host a PV has a single type recorded.

2.7.6 Physical properties of papillomaviruses.

Papillomaviruses belong to the family *Papovaviridae*. Papillomaviruses are small, non enveloped icosahedral virions, range in size from 52 to 55 nm and appear spherical in negatively stained preparations. They contain 72 capsomeres arranged on a T=7 surface lattice. The 12 capsomeres of the vertices contact five others and the remaining capsomeres contact six others. Two viral genes, L1 and L2, code for the peptides that comprise the capsomeric structural proteins. The L1 protein (MW 53,000 - 59,000) is the major component, accounting for 80% of total viral protein, and L2 (MW 70,000) provides the minor protein component (Pfister and Fuchs, 1987). The L1 protein, expressed in recombinant vaccinia virus, was shown to self-assemble into replica viral structures and no physical differences between the L1 and the combined L1 and L2 capsids could be demonstrated in this study (Hagensee *et al.*, 1994). Most papillomaviruses have a buoyant density of 1.34 g/ml in CsCl and a guanine to cytosine ratio of approximately 42% (Pfister and Fuchs, 1987).

Papillomaviruses are resistant to acids, ether and other lipid solvents, may survive for relatively long periods in the environment and wart-like papillomaviruses are frequently acquired by indirect contact via fomites. In spite of this inherent stability many papillomaviruses, human genital types in particular, rely on intimate contact between susceptible hosts for transmission (Shah and Howley, 1990).

2.7.7 Organisation of the genome.

Papillomaviruses contain approximately 8 kilobases (range 7.2 - 8.4) of double stranded, covalently closed DNA (Murphy *et al.*, 1995; Jarrett *et al.*, 1984; Isegawa *et al.*, 1994). All of the coding sequences are located on one strand and are transcribed by host-cell polymerases in the nucleus. The main model for papillomavirus transcription is BPV-1 in which the genes are subdivided into two functional groups based on temporal events in the replication cycle. Early genes are transcribed shortly after infection and code for the early proteins, E1 and E2, which sponsor interaction between the host-cell polymerases and the origin of papillomavirus replication (Chow and Broker, 1994; Lambert, 1991). Early genes or gene products can be identified in tissue sections by hybridisation and immuno-labelling techniques respectively. These processes occur in basal cells and cells which are in the early stages of differentiation (Howley, 1990; Schneider, 1987). Late genes are transcribed from a single promoter which is active in productively infected keratinocytes. Late gene products, structural proteins L1 and L2, for encapsidation of infectious papillomaviruses are produced by differential splicing and can only be demonstrated in the terminally differentiated cell.

2.7.8 Transformation.

Early proteins are essential to the transformation process and interact directly with regulatory elements of the cell cycle or with regulatory sequences of the host-cell genome (Stöppler *et al.*, 1994). In contrast, with a single promoter for late genes, there are at least six promoters for the early genes, three of which are integrated into other early genes and three are located in non-coding regions. The combination of multiple promoters and a variety of splicing patterns gives rise to a large range of mRNAs. The transcription of a proportion of these gives individual papillomaviruses a number of mechanisms with which to interact with host-cell cycle processes, the end result of which

is transformation, cell immortalisation and carcinogenesis (Pfister and Fuchs, 1987; Stöppler *et al.*, 1994). Papillomaviruses, therefore, use a variety of transformation strategies to alter the orderly differentiation of cells in squamous and mucosal epithelia (Eriksson *et al.*, 1994; Campo, 1992). For example, E7 proteins of human papillomaviruses have amino acid homology with other DNA tumour virus oncoproteins. One such oncoprotein (adenovirus E1A protein), and E7 by comparison, contains zinc finger motifs for interaction with genetic elements (retinoblastoma-susceptibility gene product) controlling events of the cell cycle (Stöppler *et al.*, 1994).

2.7.9 Immune response to papillomaviruses.

The immune responses to papillomaviruses are complex and reflect the fact that mature virions are assembled in the outer layers of differentiating epithelia. Papillomavirus infections are characterised by insidious onset and chronicity with any neoplastic consequences taking months or years to develop. These viruses are often weakly immunogenic and this phenomenon can be considered in terms of a high degree of co-evolution and adaptation between the host and the virus (Tindle and Frazer, 1994).

The intact virion displays type-specific, outer capsid epitopes and antiserum raised in rabbits has been used for serotyping human and bovine isolates (Tindle and Frazer, 1994; Jarrett *et al.*, 1984). Detergent disrupted virions display an inner capsid epitope which is group-specific and cross-reacts with many papillomavirus isolates. Labelled cross-reactive antibodies have been used to identify and locate papillomaviruses in tissue sections (Schneider, 1987). Neutralising antibodies to BPV-1 were demonstrated by titration on susceptible skin sites in the horse and by inhibition of transformation of susceptible rodent cell cultures (Olsen, 1987). Antibodies have been shown by precipitation, complement fixation or haemagglutination inhibition to be type-specific (Olsen, 1987). Transformation inhibitors have been reported in sera from calves with regressing fibropapillomas (Meischke, 1979a). Absorption of these sera with BPV suspensions did not remove transformation inhibition whereas absorption with suspensions of BPV-transformed cells or BPV-induced tumour cells did. This provided evidence for antibodies to other viral proteins or virus-induced cell products necessary for the transformed phenotype.

The viral early proteins and virus-altered cell products are more available to the host immune system both temporally and spatially. Antibody responses to the early proteins have been identified in numerous studies of HPV immune phenomena (reviewed by Tindle and Frazer, 1994). It is apparent that these responses, based on interactions between recombinant or synthetic peptides and sera, are not sufficiently clear cut for a protective role to be ascribed to the antibody response or for any one assay to provide prognostic information. Antibodies to viral structural proteins are not believed to play a significant role in papilloma regression due to the protected peripheral location of the virus particles. In serological assays of reactivity to capsid proteins there is a poor correlation between level of antibody and the disease state, with one study of HPV-6 showing similar reactivity for affected and control groups (Tindle and Frazer, 1994).

Cell-mediated immunity is regarded as the more important mechanism of papilloma regression with mononuclear inflammatory cells characterising the chronic reaction. Cell mediated responses have been demonstrated in leucocyte migration assays and suggested by the increased size and incidence of cutaneous papillomas when T-cell functions are suppressed (Shah and Howley, 1990). A case report of persistent papillomatosis in a yearling bull, prompting an investigation of its immune status, found only cell-mediated immunity to be deficient (Duncan *et al.*, 1975).

2.10 AIMS AND SCOPE OF THESIS

Prior to the commencement of this research there were no reported or anecdotally described diseases of wild or captive possums in New Zealand that were shown or postulated to have a viral aetiology. This thesis describes attempts that were made to detect, isolate and characterise any viruses that might be present in possums in N.Z.

Investigations would be conducted to identify a possum-specific virus with properties suitable for molecular modification towards humane biological control purposes. The ideal virus, it was reasoned, would cause mild disease or subclinical infection with a long infectious period, would not already be widespread in the population, would persist in the environment, be readily transmitted by the somewhat minimal social activities of possums and be amenable to genetic manipulation. However, any viral agent causing significant disease and/or falling predictably short of other expectations would be studied and assessed with specific reference to the desired objectives.

The most likely way to isolate new viruses from a relatively unstudied species was to define and develop methods for cultivation of possum cells and related, potentially permissive, marsupial cell lines. The process of establishing primary cell cultures from possums would allow an opportunity for adventitious or 'latent' viruses to replicate unhindered by host immune surveillance. A supply of possum derived primary cells and stable cell lines would hopefully provide a sensitive and robust *in vitro* cultivation system for viruses present in possum tissue suspensions. The development of a survey protocol for the isolation of possum viruses based on tissue culture is described in chapter three.

Two potential sources of possum viruses were identified. Presumed healthy, wild-captured possums could supply a wide range of fresh tissues, including swabs of tissue fluids from the pharyngeal and cloacal regions, that potentially harboured endogenous possum viruses. In this category were possums hypothesised to have subclinical infections or to be convalescing from overt disease. Stress associated with captivity was expected to allow such disease processes to further develop or for latent viruses to recrudesce. Persistently infected animals with low level excretion of viruses may excrete substantially increased numbers when subjected to the stresses of capture and

confinement and latently infected animals may begin to shed infectious agents that are otherwise suppressed by immune mechanisms.

A second potential source of possum viruses came in the form of fresh pathological specimens from possums with lesions which paralleled those associated with virus infections in other species or possums with a clinically recognisable illness. For example, a possum with a papilloma on the tail was submitted for further investigation. The resulting confirmation of a new papillomavirus type associated with possums is described in chapter eight. Following a report that a disease process in possums in the Rotorua district was causing behavioural changes and death, a thorough field investigation with appropriate laboratory back up was instigated. The findings from this investigation are reported in chapter seven. Also in this latter category, tissues from possums with clinically confirmed WPD (2.2.1) were submitted for investigation. Description of this disease process and attempts to isolate and characterise the causative agent are presented in chapters four and five. A series of experiments to determine the routes of transmission of WPD are the subject of chapter six.

Chapter 3

POSSUM VIRUS SURVEY

3.1 INTRODUCTION.

At the time of commencing this study three approaches had been taken or were under way to identify viruses of possums in New Zealand. A serological survey had identified antibodies in possum sera which reacted with the group-specific antigen of mammalian adenoviruses (Rice *et al.*, 1991). This provided evidence that there were adenoviral infections in wild possums and a subsequent survey of possum intestinal contents for virus particles using electron microscopy (E.M.) demonstrated typical adenovirus particles in six out of 100 samples (Rice and Wilks., 1996). In the E.M. survey, coronaviruses or coronavirus-like particles were detected in five and 13 samples respectively. Enveloped herpesvirus particles and herpesvirus capsids were identified in two samples.

An attempt to culture possum viruses had been conducted in 1991 but no viruses were isolated (Horner, 1992). In that study, pooled tissue homogenates were prepared, from dead possums supplied by hunters, and inoculated by standard methods onto three marsupial cell lines. Two of these cell lines, potoroo kidney cells (PTK2) and opossum kidney cells (OPK), were derived from marsupial species distantly related to *T. vulpecula*. A third cell line, possum kidney cells (PK), derived from *T. vulpecula* was also developed during the course of the study.

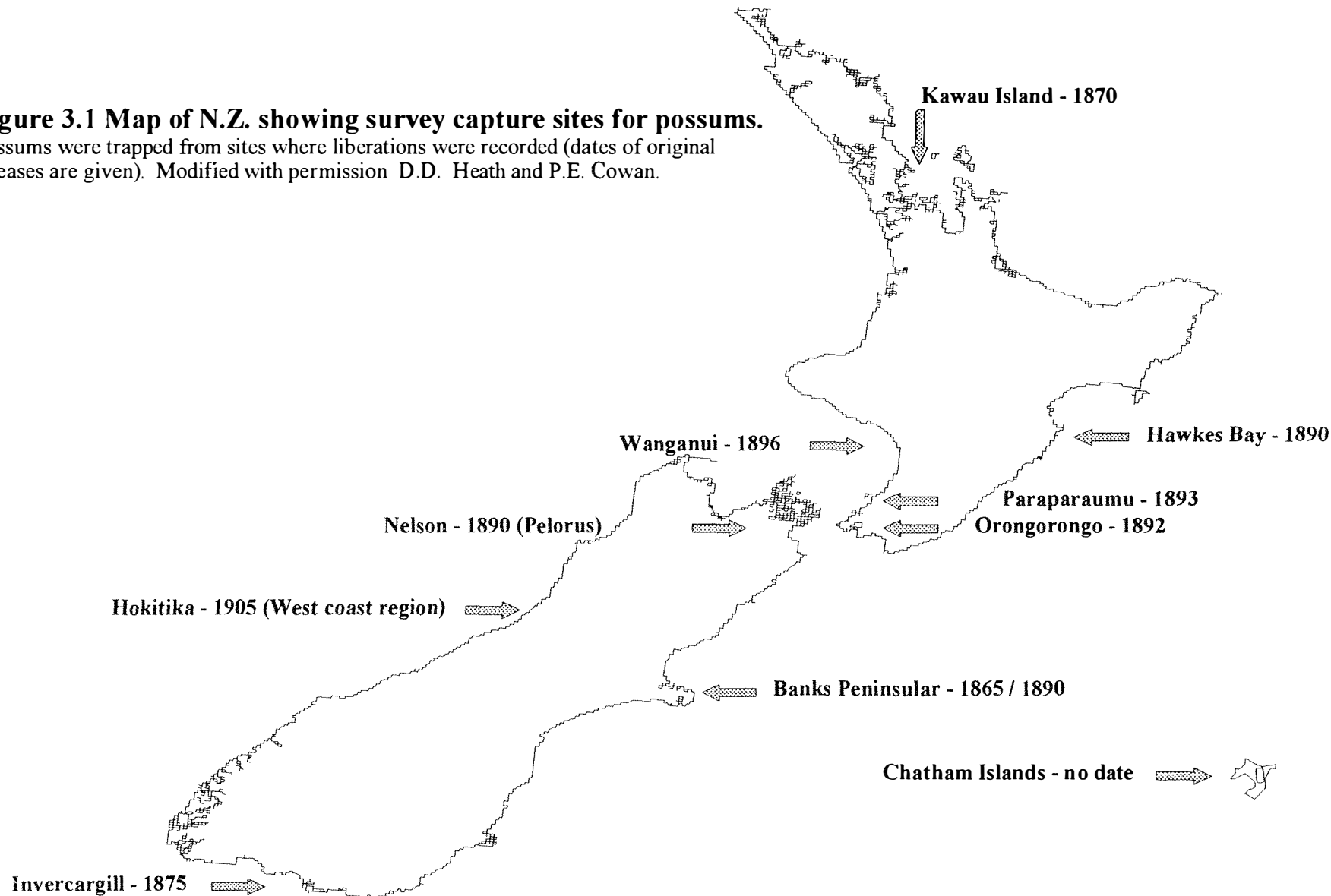
In light of the above work there was good evidence that possums possessed a range of endemic viruses but that suitable methods had not yet been used to propagate them *in vitro*. It was suggested (Horner, 1992) that the negative findings were more a comment on the methodology employed than a reflection of the viral burden of possums and more sophisticated techniques, namely co-cultivation and autogenous (primary) cell culture were recommended. It was also possible that the failure of the Horner study to isolate

viruses was due to the less than optimal state of the tissues when processed rather than non-permissive indicator cells. Therefore, co-cultivation of live cells, harvested from a range of organs of recently killed possums was the basis of the present study. Co-cultivation of live cells with a range of potentially permissive cell monolayers would give, it was reasoned, the best chance of propagating *in vitro* any viruses that were present in the tissues at processing whether they were undergoing productive infection or latent at the time the tissues were prepared.

The survey to identify viruses using tissue culture was run in conjunction with, and supplied tissues for a collaborator who used a molecular biological approach to screen possum tissues for viral nucleic acids using the polymerase chain reaction (PCR). This complementary approach aimed to identify samples containing nucleic acid sequences of viruses. Primers were initially designed to target conserved sequences of adenoviruses, herpesviruses and retroviruses. These two projects were performed in conjunction with a larger survey to document the parasite burden of the New Zealand possum population during this period. Possums were supplied to both research groups by Landcare Research. The capture areas for the survey animals were chosen from historical data on the original sites for possum liberation throughout New Zealand (figure 3.1). In choosing to sample a wide variety of mainland sites and offshore islands it was hoped that both imported genetic diversity and local adaptation events could be included in the pool of animals examined. There is evidence to suggest that the spread of possums from local release sites was limited by geographic barriers and that genetic diversity in N.Z. possum populations may have been partially maintained (Triggs and Green, 1989; Taylor *et al.*, 1998).

Figure 3.1 Map of N.Z. showing survey capture sites for possums.

Possums were trapped from sites where liberations were recorded (dates of original releases are given). Modified with permission D.D. Heath and P.E. Cowan.



3.2 MATERIALS AND METHODS.

3.2.1 Marsupial cell lines.

Three marsupial cell lines were used for all standard isolation protocols. Possum kidney (PK) cells at pass 28, Potoroo kidney (PTK2) cells at pass 75 and Opossum kidney (OPK) cells at pass 45 were kindly supplied by AgResearch, Wallaceville. Potoroo kidney and OPK cells were originally sourced from the American Type Culture Collection (ATTC) as ATTC-CCL-56 and ATTC-CRL-1840 cell lines respectively. All cells had an epithelial morphology but varied with respect to growth characteristics and ease of maintenance.

During the course of the study, two morphologically identical cell lines from the reproductive tract tissues of two female possums were developed and added to the protocol to replace the PK cells (3.2.7, 3.3.4). The two female possum reproductive tract cell lines (PRT-1 and PRT-2) showed similar growth characteristics to each other and to the PK cells and were used at passage 5 - 15.

3.2.2 Cell culture.

(i) Media : All media, reagents and recipes used for cell culture work are detailed in appendix one. Cells were initially cultured in a growth medium (GM) comprising minimum essential medium (MEM, Sigma CELL CULTURE™) supplemented with 10% foetal bovine serum (FBS), 10% lactalbumin hydrolysate containing Earle's salts (ELH, Sigma CELL CULTURE™), 10% tryptose phosphate broth (TPB) and 1% penicillin, streptomycin, kanamycin (PSK). The pH of the medium was adjusted to 7.0 - 7.2 and 1% L-glutamine or GlutaMAX™ (Gibco, BRL) and 1% PSK was added when media had been stored for longer than one week. All cell lines grew adequately with reduced FBS (5%), which became standard for the GM. Maintenance medium (MM) contained 1% FBS. All other additives were constant.

(ii) Cell passage : Briefly, monolayers were washed with two changes of phosphate buffered saline (PBS), pH 7.2, and incubated at 37 °C with 1, 2 or 4 mls of 0.05% antibiotic, trypsin, versene (ATV) for small (25 cm²), medium (80 cm²) or large (175 cm²) flasks respectively. Following detachment the trypsin reaction was stopped by the addition of 10 mls of media containing 5% FBS. Viable cells were counted (Neubauer

haemocytometer) using trypan blue dye exclusion and sub-cultured at a concentration of approximately 1×10^5 live cells per ml of media. For small, medium and large flasks 8 - 12, 20 - 30 and 50 - 70 mls of cell suspension was used respectively. The PK, PRT-1/2 and PTK2 cells were split at a ratio of 1:3 and the OPK cells split at a ratio of 1:4.

(iii) Cryo-preservation of cells : Cells were trypsinized with ATV as above and centrifuged at $300 \times g$ for 10 minutes. The cell pellet was resuspended in 1-2 mls of MEM and a viable cell count performed. Dimethyl sulfoxide (10%) and FBS (20%) were added to make a final live cell concentration of $0.5\text{-}2.0 \times 10^7$ cells/ml in MEM. Cells were aliquotted (1 - 1.5 mls) into cryo vials (Nunclon, Intermed), frozen to -70°C for 12-24 hours in a cooling chamber containing isopropyl alcohol and transferred to liquid nitrogen. To resuscitate stored cells cryo vials from liquid nitrogen were rapidly thawed in a water bath at 37°C and transferred to an equal volume of growth medium at 37°C . A live cell count was performed and cells were seeded at $0.5 - 1.0 \times 10^6$ cells per ml in small or medium flasks.

(iv) Laboratory equipment : Cell culture procedures were carried out in a laminar flow cabinet (Gelman biohazard) using aseptic tissue culture techniques. Cells were incubated in a humidified atmosphere containing 5% CO_2 (QUEUE® cell culture incubator). Throughout the study period bench centrifugations were performed in either an IEC Centra-4B (International equipment company) or a Centaur MSE (Kempthorne medical supplies). Nunc tissue culture flasks were used for maintenance and passaging cells. An Olympus IMT-2 inverted microscope equipped with phase contrast and capable of 40, 100, 200 and 400 x magnification was used for all critical evaluations of cultured cells. An Olympus OM2, manual SLR camera and Kodak (Ektachrome 64 Tungsten) film was used for all photographic recordings.

3.2.3 Experimental animals.

(i) Source of experimental animals : For the survey, up to 12 possums per batch were live-trapped from ten geographically separated locations and couriered by road or air directly to Massey University Small Animal Production Unit (SAPU)(Table 3.1). On arrival at SAPU possums were examined for signs of transport stress, disease or physical

injury. Where an animal showed clinical signs for which a viral infection was suspected, tissues were collected for co-cultivation within 24 hours. The welfare of possums showing clinical signs of disease or injury was considered in any decision to prolong life.

Table 3.1 Sources of possums for survey.

Source of possums number supplied ()	Series ID	Source of possums number supplied ()	Series ID
Hawkes Bay (9)	0	Banks Peninsula (12)	500
Paraparaumu (12)	100	Pelorus, Nelson (10)	600
Wanganui (12)	200	West coast region (11)	700
Orongorongo (12)	300	Invercargill (12)	800
Kawau Island (10)	400	Chatham Island (12)	900

On occasion, minor injuries and infections were treated with broad spectrum injectable antibiotics (Clavulox®, SmithKline Beecham, at a dose rate of 20 mg/kg or Baytril® 2.5%, Bayer, at a dose rate of 0.2 ml/kg). Individuals under treatment were identified with sprayline stock marker or separated from the main group for observation. Possums were assigned a series number according to their origin and an individual number reflecting the order in which they were killed to supply tissues for the survey. For example, possum 207 was the seventh possum processed from Wanganui (table 3.1).

(ii) Housing and feeding : Possums were housed in a group cage (3.0 x 4.5 x 2.4 m) made from steel pipe and wire netting. The enclosure had a well drained, concrete floor and protection from the weather was provided by galvanised iron cladding on one end wall and half of the adjacent side walls and roof. At the sheltered end of the cage, a free standing set of three metal shelves (rigid unit with shelf dimensions 2.5 x 0.5 m and height 1.5 m) was attached to the end wall and used to suspend individual plastic nest boxes (250 x 250 x 480 mm). Plastic nest boxes were also secured to walls at the sheltered end of the cage. The top metal shelf provided a platform from which wooden, aerial runways were constructed from pine tree branches and these were joined to tree trunks secured from floor to roof. A large wooden nest box (700 x 400 x 400 mm) and a smaller wooden box (400 x 300 x 250 mm) were later secured to the top shelf.

The cage was cleaned in the morning, when the possums were relatively inactive. Possums were fed in the late afternoon when they were more easily aroused. The diet was based on commercial rabbit pellets, supplied *adlibitum*, with supplementation of fruit and vegetables in season. For variety, on an irregular basis, bread, cereals, willow leaves and sprouted peas were fed. Several feeding stations and water bowls were provided.

(iii) Possum handling and treatment : Possums in the 300 - 500 series were treated with 3 mg of triamcinolone acetonide (Vetalog® LA, Ciba-Geigy) by intramuscular injection upon arrival at SAPU. Possums in the 600, 800 and 900 series were similarly treated 7 - 14 days prior to euthanasia. Drug administration appeared to cause minimum stress to possums and could often be done in the morning without arousal. Where necessary, possums were restrained with the aid of a heavy sack to hold the tail. Intramuscular injections were administered in the caudal thigh region using a 23 g x 1" needle. Occasionally, for a highly aroused animal, it was necessary to administer the tranquilliser from a syringe mounted on the end of a dowel prod using a 20 g x 1/2" needle. Ketamine, 100 mg/ml (Phoenix pharmaceutical distributors, Auckland), at a dose rate of 15 - 50 mg/kg provided chemical restraint and diazepam, 5 mg/ml (Pamlin, Parnell Laboratories, Auckland) at a dose rate of 0.5 - 2.0 mg/kg provided additional sedation when required.

3.2.4 Tissue collection.

(i) Preparation of possums : Possums were restrained by administration of ketamine, weighed, carefully examined and shaved at the xiphisternum. The area was swabbed with surgical spirit and an 18 g x 1 1/2" needle used to collect 20 ml of blood by cardiac puncture into anticoagulant (EDTA) and plain vacutainers. The possum was euthanased by intracardiac, pentobarbitone overdose. Pharyngeal swabs were collected and cloacal swabs taken if the external orifice was sufficiently free of faecal contamination. The possum was submerged to the neck in soapy water and wet thoroughly. The cranial, ventral surface of the neck and the area between the mandibles was shaved. The possum was transferred to a dissection board where it was tied by all four legs and upper jaw in dorsal recumbency.

(ii) Dissection technique : The cranial ventral neck region was doused with surgical spirit and using sterile instruments, a midline incision was made in the cranial neck region. The skin was reflected to each side with care being taken to avoid fur entering the site. Instruments were washed free of tissue and blood in water, transferred to surgical spirit and flamed before each onward step in the dissection process. In this manner the salivary glands were collected into transport medium and held at 4 °C. The tongue was excised, the pharynx and larynx reflected and the retro-pharyngeal lymph nodes and tonsils collected into transport medium (appendix one).

The ventral incision was extended to the pubis and the skin reflected from the entire ventral surface of the animal. The abdomen was lightly doused with surgical spirit, opened and the viscera reflected to one side. The mesenteric lymph nodes were collected and pooled with the retro-pharyngeal lymph nodes. Sections of spleen, kidney, liver, ovary or prostate were collected into transport medium and the liver and viscera reflected from the abdomen. Caecal and jejunal contents were collected separately into sterile specimen containers at the end of dissection procedures. The chest cavity was entered through an opening in the diaphragm and a piece of lung tissue collected.

For possum batches 600 - 900, neurological tissues were also collected. To access the brain, the jaw was removed, skin and soft tissues dissected clear of the skull and the occipito-atlantal joint dislocated. The cranium was run through a band saw along the sagittal midline and the trigeminal ganglia identified and freed from bone. The lumbar spine was dissected free from skin and excess soft tissue and the dorsal spinous processes and adjacent bone partially removed using a band saw. The spinal canal was opened with bone cutters and the dorsal root ganglia from L2 to L6 removed by sharp dissection.

(iii) Storage of samples : All tissues (except those listed below) were held at 4 °C and processed as soon as possible on the day of collection. Pharyngeal and cloacal swabs were collected into 1 - 2 mls of a maintenance medium (MM containing 2% FBS, 2% PSK and 1% Fungizone®) for storage at -70 °C. Gut contents were stored at -70 °C. Clotted blood was held at 4 °C prior to separation and storage of serum at -20 °C.

3.2.5 Co-cultivation.

(i) Preparation of cell monolayers : Initially, the number of tissues processed was tailored to fit onto one 24 well multiplate, representing 8 wells for each of the three cell lines (PK, OPK, PTK2). Cell monolayers were prepared on the day prior to processing a possum in order to achieve an 80 - 100% confluency at the time of inoculation.

(ii) Preparation of single cell suspensions : Five tissues (liver, kidney, lung, prostate and lymph node) and four methods were assessed for the preparation of single cell suspensions to use for co-cultivation. These methods included a hand held, high speed tissue homogeniser (Dremel), low speed physical disruption and maceration of tissues (Colworth Stomacher 400), a standard trypsinisation procedure and fine mincing with scalpel blades. A fine mincing and elution protocol (described below) was chosen because of simplicity and relatively high live cell yields (data not presented).

(iii) Tissue preparation and monolayer inoculation : A 1 cm³ portion (liver, kidney, spleen, lung and prostate), or the entire tissue of smaller samples (ovary, lymph node and salivary gland) was minced finely with scalpel blades and 2 ml of MEM containing 2% FBS and 1% PSK used to elute individual cells and small tissue fragments. Approximately 300 µl of each tissue suspension was added to each of 3 wells containing OPK, PTK2 or PK cell monolayers. The inoculated monolayers remained in 2 ml of GM and were incubated for one week at 36 °C.

(iv) Observation of cell cultures : For the first passage, control wells and monolayers not fully obscured by tissues (control, salivary gland, ovary and lymph node) were checked once at 3 - 4 days post-inoculation (pi) and once prior to freezing (7 days pi). During the second passage monolayers were more visible (wells with liver, kidney, lung, spleen and prostate samples were partially obscured) and were checked every 2 - 3 days. Monolayers were observed every 2 days for the third passage.

(v) Passaging of co-cultures : After one week of incubation co-cultures were subjected to two cycles of freezing (-70 °C) and thawing prior to passage. Media and monolayer debris was mixed and 200 µl of each cell suspension was transferred to the

corresponding well of the second or third plate, containing 80 - 100% confluent OPK, PTK2 and PK cell monolayers, and incubated as described above. For possums in the 500 - 900 series incubation of cultures continued following haemadsorption and replacement of GM with MM medium at the end of the third passage. Multiplates were observed twice weekly with a partial change of MM following observation and kept for one month. If no cytopathic changes were observed during this period the plate was discarded.

3.2.6 Methods for detection of viruses.

In order to detect a cytopathic effect (CPE), each of the three passes was maintained for at least one week and, where possible, the third pass was extended, with media changes, for one month following haemadsorption. All third pass cultures were tested by haemadsorption and monolayer cultures in slide chambers (or on glass discs) were fixed, stained with haematoxylin and eosin (H&E) and examined.

(i) Haemadsorption (HA) : Chicken, guinea pig and human type "O" red blood cells (RBCs) were collected by venepuncture or cardiac puncture, transferred into four volumes of Alsever's solution and stored at 4 °C for a maximum of one week. Suspensions of RBCs were counted using a haemocytometer (chicken) or haematology analyser (Cobas Minos® Vet, ABX Hematologie) appropriately adjusted for RBC size. Following enumeration, RBCs from each species were pooled to make a mixed RBC suspension of numerically equal proportions. Pooled whole blood (total volume 1 ml) was washed in 10 volumes of PBS, the resulting suspension centrifuged (700 x g for 10 min) and the supernatant discarded. Washing and centrifugation was repeated and the final supernatant removed. A 0.5% RBC suspension was prepared by adding 500 µl of packed cells to 99.5 ml of PBS (Wiedbrauk and Johnston, 1993).

The HA test was performed five days following inoculation. An aliquot (0.3 ml) of the 0.5% mixed RBC suspension was added to each well, the plate incubated for 20 minutes at 37 °C and the test read at 100 x magnification. The negative control was the non-inoculated monolayer for each cell type on each multiplate. Positive controls, Madin-Derby Bovine Kidney cells incubated with bovine para-influenzavirus-III for varying

times, established the range of haemadsorption phenomena but were not run routinely. In the event of haemadsorption or ambiguous phenomena the passage was repeated to include a range of positive controls.

(ii) Chamber slides : Cell monolayers were prepared in eight-well chamber slides (Tissue Tek, Nunc). At early confluency, the cell monolayers were inoculated with 50 - 75 μ l of cell suspension from passage two cultures, incubated (36 °C for 4 - 5 days), fixed with Bouin's fixative for 12-18 hours followed by 70% ethanol for 1 hour, freed from plastic chamber housing and stained with haematoxylin and eosin (H & E). Chamber slide wells were examined under a light microscope at 100 and 400 x magnification.

(iii) Culture surface disc inserts for multiplates : Possum and chicken embryo primary cells performed better on circular glass or Thermanox® (Nunc, Inc. Illinois) multiplate inserts used as culture surfaces inside 24 well plates than in chamber slides. Appropriately inoculated and incubated monolayers were fixed *in situ* (as above), removed with a hooked, 18 g needle and forceps, stained with H & E in a special rack and examined as above.

3.2.7 Primary cell lines.

(i) Enzymatic digestion - trypsin : Several attempts were made to establish possum cell lines by enzymatic disaggregation of kidney cortex, lung, prostate and lymph node. Tissues were collected aseptically (preferentially from joeys), minced finely with scalpel blades, washed in two changes of PBS, transferred to a sterile trypsinising flask containing a magnetic flea and 15 volumes of 0.135% trypsin solution per volume of tissue. Tissues were agitated at 37 °C on a magnetic stirrer for 30 minutes. Digested tissues were decanted from the side arm of the flask and filtered through sterile gauze or mesh into 1 ml of FBS in a centrifuge tube. Cell suspensions were centrifuged at 300 x g for 10 minutes and supernatants discarded. Cell pellets were resuspended in residual liquid and then in 10 mls of primary growth medium. Live cell counts were performed and the cells plated out at 2×10^6 cells/ml in small flasks containing primary medium (PM).

Primary chicken embryo fibroblasts (PCEFs) were prepared following a similar protocol. Embryonating eggs were candled at 9 - 11 days and viable embryos removed from their shell aseptically and decapitated. Extremities were removed and the embryos minced and washed as above. The embryo was disaggregated using twenty mls of 0.135% trypsin (30 minutes at 37 °C) and passed into the centrifuge tube through a gauze that had been pre-wet with 1 ml of FBS. The PCEF cell suspension was centrifuged and resuspended in chicken GM (MEM supplemented with 10% FBS, 10% TPB and 2% PSK) and plated (1×10^6 cells/ml).

(ii) Enzymatic disaggregation - Dispase : After standard mincing and washing in PBS, cortical tissue from possum kidneys was transferred to a trypsinising flask with 35 - 50 ml of Dispase[®] solution (Gibco, BRL). The kidney was digested overnight for 12 - 18 hours at 4 °C with gentle agitation. The tissue suspension was then centrifuged at 300 x g for 10 minutes, the supernatant discarded, the cell pellet resuspended in growth media and live cells enumerated. Cells were seeded into small flasks at 2 - 6 x 10⁶ cells per ml of PM depending on the desired time to confluency.

(iii) Explant techniques : Possum cells were grown from fragments of tissue explanted onto a culture surface (Pye *et al.*, 1977). Tissues were collected under sterile conditions, minced in a petri dish and washed with PBS. Small fragments (~ 0.5 - 1.0 mm³) were placed onto an etched plastic culture surface (small flask or six well multiplate). Tissue fragments were bathed in 0.3-0.5 mls of chick embryo extract (Gibco, BRL) and held at 4 °C for approximately 30 minutes. Chicken plasma (0.25 or 0.5 ml) was added to each explant well or flask respectively and incubated at 37 °C for 30 minutes. Chick extract and plasma was partially removed and 3-6 mls of primary medium (PM) carefully added to each well or small flask. The medium formed a soft clot over the explant cultures which were examined twice weekly and given partial medium changes once the clot began to break down. Explant primary cultures were routinely supported with PM and filtered conditioned media (FCM). Possum primary cultures prepared using other methods were supported with 12-15% FBS and FCM when available.

3.2.8 Expansion of protocol.

(i) Rationale: No viruses were detected from the first six series of possums (000-500). It was decided to include explants of a selection of neurological tissues to look specifically for latent alpha-herpesviruses using the same marsupial cell lines as described in section 3.2.1. As this required a second multiplate per possum processed, extra samples (tonsil, buffy coat and intestinal contents) were added to the protocol to fully utilise this plate.

(ii) Additional tissues : Lumbar dorsal root ganglia, trigeminal ganglia, brainstem and tonsils were collected and stored in transport media until other tissues had been processed. These tissues were then minced finely and co-cultivated with increased levels of antimicrobials in the MM (2% PSK, 0.5% Baytril®, 0.5% Fungizone®). The buffy coat was carefully removed from the RBC pellet after centrifugation at 1000 x g for ten minutes. Contaminating RBCs were lysed by dilution in five volumes of sterile distilled water (dH₂O) for 45 seconds. Isotonicity was restored by addition of an equal volume of 0.3 M NaCl and the white blood cells (WBCs) pelleted by centrifugation at 300 x g for ten minutes. The cells were resuspended in 600 µl of MM and 200 µl of the WBC suspension co-cultivated with each cell line as above 3.2.5(iii).

Large and small intestinal contents samples were prepared as 10% suspensions in PBS and mixed by agitation (vortex) for 5 minutes, allowed to settle, mixed a second time, centrifuged at 1800 x g for 20 minutes and the supernatants filtered through a 0.22 µm membrane. Filtered intestinal suspensions were titrated once on each monolayer to determine the threshold for toxic effects. Based on these results a 0.25 ml aliquot of the 10% suspension was cultivated with each cell line.

(iii) Passage of additional tissues : Three blind passages were performed as previously described (3.2.5) and haemadsorption and chamber slide procedures completed (3.2.6). The major difference for the second co-cultivation (CC-II) multiplate was that cultures were not frozen and thawed between passages and that tissue fragments, as well as media and monolayer cells, were transferred by sterile forceps.

(iv) Explant primary (EP) culture : Fragments of kidney, lung, tonsil, lymph node, ovary or prostate were explanted into six-well multiplates in order to establish primary cells (Pye *et al.*, 1977). Outgrowth from tissue fragments was supported by regular changes of PM as previously described (3.2.7). After several days, and up to three weeks following establishment, primary cell growth from fragments became maximal (occasionally confluent monolayers). As a viral CPE could not be differentiated from routine primary cell deterioration, all primary cell sheets and monolayers, including those that had progressed from the expansion to the deterioration stage, were stored (-70 °C).

(v) Explant primary culture passage : Primary cell growth from competent tissues was treated as a first passage. When stored primary cultures could be batched, 24 well multiplates of OPK, PTK2 and PRT-1/2 cells were established (3.2.5), primary cultures were thawed, frozen and thawed and 200 µl aliquots passaged as for a second passage of the standard protocol (3.2.5). A third passage, haemadsorption and chamber slide examination procedures were completed as previously described (3.2.6).

3.2.9 Sensitivity of co-cultivation for detection of viruses.

(i) Preparation of virus : A 0.2 ml aliquot of Macropod herpesvirus-1 (MaHV-1) containing 3×10^5 TCID₅₀/ml was diluted 1000-fold in maintenance medium and a 0.5 ml aliquot used to infect confluent OPK cells in a medium flask. Following detection of CPE the flask was frozen and thawed, remaining adherent cells scraped into the medium and the cell suspension mixed for two minutes, divided into aliquots and stored (-70 °C).

(ii) Experimental protocol : Twenty-four-well multiplates of OPK cells were prepared. The ability of MaHV-1 to replicate in OPK cells in the presence of concentrated tissue inocula was tested using five possum tissues that obscured cell monolayers on first passage (liver, spleen, kidney, lung and brain) and one tissue that was non-obscuring (salivary gland). One tissue was tested per multi-plate. Each tissue (1 cm³) was minced and eluted with 2 mls of MM and 10-fold serial dilutions prepared with MM. One aliquot of the stored MaHV-1 was thawed at room temperature and serial 10-fold dilutions prepared in MM.

Four combinations of the tissue under test and/or virus dilutions were titrated (Table 3.2). Row one was a titration of tissue and virus in the same well. A standard aliquot (300 µl) of each tissue dilution (10^0 - 10^{-5}) was added to each monolayer followed immediately by 200 µl of the virus suspension or its appropriate dilution (10^0 - 10^{-5}). Row two was a constant aliquot (300 µl) of concentrated tissue (10^0) followed immediately by 200 µl of each virus dilution (10^0 - 10^{-5}) as above. Row three was a tissue titration (10^0 - 10^{-5}) and row four a virus titration (10^0 - 10^{-5}).

The monolayers were incubated (36 °C) and read at 24 hours and six days post-inoculation. Multiplates were frozen and thawed twice after one week of incubation and a 200 µl aliquot from each well transferred to the corresponding well of the subsequent multiplate (3.2.6). The titre of MaHV-1 in the original suspension was calculated from row four (six replicates) using the Karber formula (Mohanty and Dutta, 1981).

Table 3.2 Ten-fold dilutions of tissue (T) and MaHV-1 (V).

Tissue dilution, Virus dilution.	Virus dilution, Tissue constant	Tissue dilution (titration)	Virus dilution (titration)
T 10^0 , V 10^0	T 10^0 , V 10^0	T 10^0	V 10^0
T 10^{-1} , V 10^{-1}	T 10^0 , V 10^{-1}	T 10^{-1}	V 10^{-1}
T 10^{-2} , V 10^{-2}	T 10^0 , V 10^{-2}	T 10^{-2}	V 10^{-2}
T 10^{-3} , V 10^{-3}	T 10^0 , V 10^{-3}	T 10^{-3}	V 10^{-3}
T 10^{-4} , V 10^{-4}	T 10^0 , V 10^{-4}	T 10^{-4}	V 10^{-4}
T 10^{-5} , V 10^{-5}	T 10^0 , V 10^{-5}	control	V 10^{-5}

T and V dilutions contain standard volumes of 300 µl and 200 µl respectively.

3.2.10 Electron microscopy.

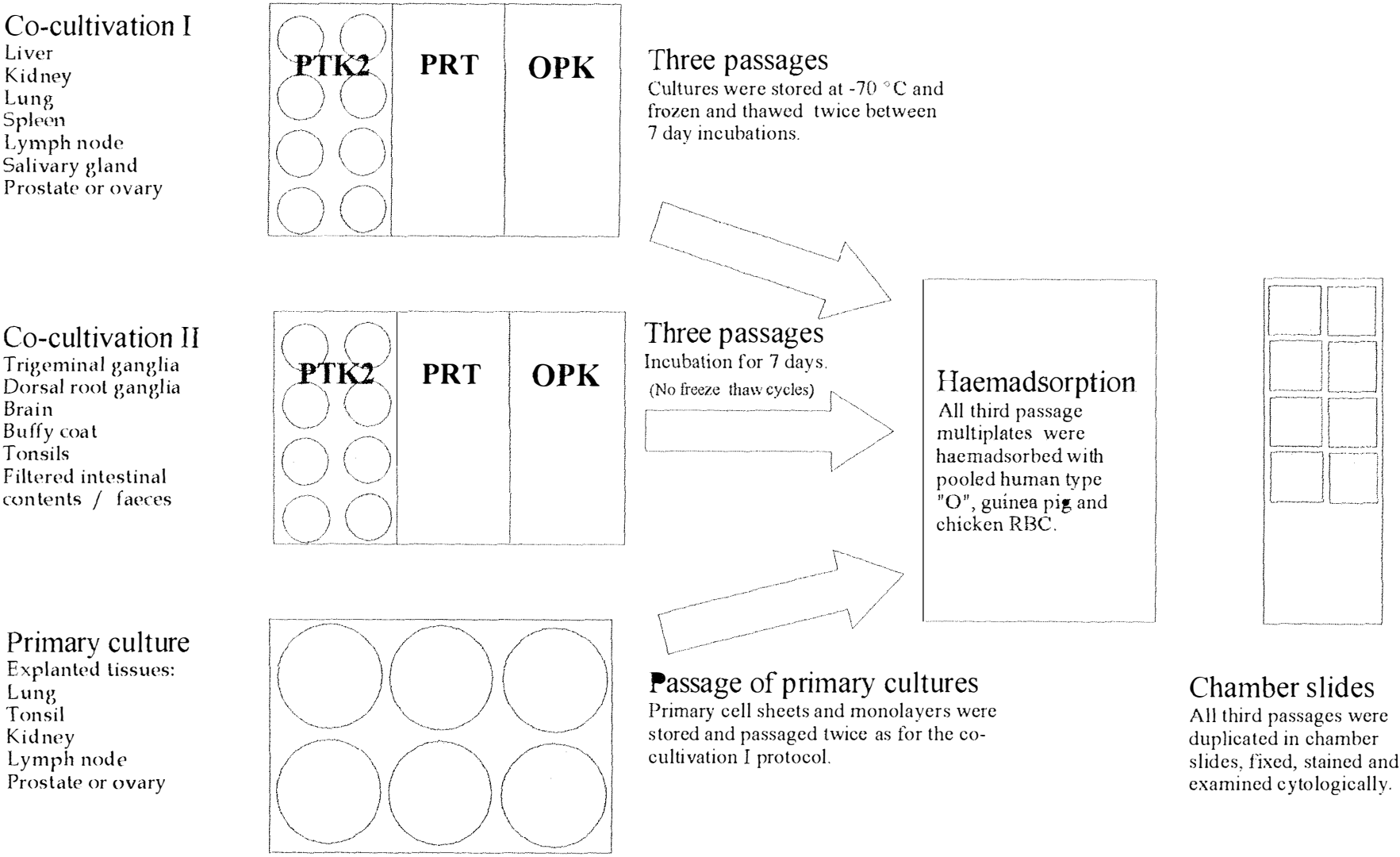
(i) Sample preparation : Suspensions (10%) of faeces and intestinal contents in PBS were prepared in 15 ml centrifuge tubes. Suspensions were mixed by agitation (vortex) for two minutes, allowed to settle, mixed again and centrifuged at $2700 \times g$ (30 minutes). Supernatants were ultra-centrifuged ($180,000 \times g$) for 2 hours over a 1 ml cushion of 45% (w/w) sucrose. Supernatants were discarded and the pellet resuspended in 50 μ l dH₂O overnight.

(ii) Negative staining : All reagents and recipes for electron microscopy are presented in appendix three. Formvar coated, 400 mesh copper grids were placed coating side down on a 30 μ l drop of 1% bovine serum albumin (BSA) for 20 seconds. Excess BSA was removed by blotting and the grid transferred to a drop of sample for 30 - 45 seconds. The grids were blotted dry, washed by floating on a drop of dH₂O and dried again. Grids were stained by floating on a drop of 2% phosphotungstic acid (pH 7.0) for 45 seconds, thoroughly dried and examined using a Philips 201c electron microscope.

Summary of tissue culture survey aims, methods and amendments.

1. Series 000 - 900 : Co-cultivation (CC-I) of liver, kidney, spleen, salivary gland, lung, retropharyngeal and mesenteric lymph node (pooled), prostate or ovary tissues as single cell suspensions and small clumps of cells.
2. Three seven-day passages of CC-I multiplates were performed (36 °C : 5% CO₂ : humidified incubator). Multiplates were frozen and thawed twice and 200 µl aliquots of each co-culture transferred to fresh, subconfluent monolayers. Multiplates were stored at -70 °C and processed in batches of five following the initial passage.
3. All third pass multiplates were haemadsorbed after 5 - 6 days of passage with pooled chicken, guinea pig and human type "O" red blood cells at 36 °C. When feasible, the third passage was prolonged following haemadsorption and monolayers were supported with twice weekly partial maintenance media changes.
4. Cytological examination of fixed, stained third passage monolayers (chamber slides).
5. Series 500 - 900 : Co-cultivation (CC-II) of brain, trigeminal ganglia, dorsal root ganglia, tonsil and buffy coat mononuclear cells. Cultivation of filtered intestinal contents. CC-II multiplates were subsequently processed as described in 2, 3 and 4 above with the exception that passages were not separated by freeze / thaw cycles.
6. Series 500 - 900 : Explant primary culture of selected tissue fragments (kidney, lung, salivary gland, tonsil, lymph node, prostate and ovarian tissue). Primary cultures were supported beyond maximal, healthy outgrowth from explants and stored (-20 °C). Initial primary cultivation of tissue fragments was considered as a first passage and two further primary explant passages (EPP), including haemadsorption and chamber slide examination, were completed (2, 3 and 4 above).
7. Commitment to establish primary and low passage possum cells to increase the sensitivity of the co-cultivation indication system.
8. Investigation of all unexpected causes of death in survey possums by post mortem, histopathology and tissue culture where appropriate. Investigation of outbreaks of disease in wild possum populations when resources available.
9. Provision for welfare aspects of housing captive possums (shelter, an adequate diet and relief from injuries or illness) while maintaining a moderate degree of stress through social crowding. Corticosteroids were given to apparently healthy possums to mimic or enhance immunosuppression caused by stress.

Figure 3.2 Flow diagram of tissue culture protocols.



3.3 RESULTS

3.3.1 Experimental animals.

(i) Initial treatment and handling : On arrival at Massey University, a number of possums were observed to have lower limb injuries presumably as a result of trapping. Baytril and Clavulox were usually effective at treating and preventing septic complications to open wounds but a small number of possums were killed for welfare reasons. Possums were routinely sedated to check and debride wounds when necessary. Profound sedation was achieved with ketamine if possums were not aroused. All possums significantly aroused prior to or by the injection procedure were less sedated for a given dose of ketamine. Diazepam aided sedation in possums aroused by injection and sedation of juveniles and young adults was more consistent when both drugs were used. Tranquillisation was adequate for the cleaning of minor wounds, venepuncture and cardiocentesis. Recovery from sedation was uneventful in all but two cases.

(ii) Maintaining possums in captivity : Possums appeared to adjust to the captive environment in 2 - 4 days although observations of nocturnal behaviour were limited. Possums ate the fresh fruit or vegetable component of the daily ration first and bread and willow leaves were readily accepted. Pellets were consumed after a period of adjustment but eating behaviours of individual possums were not recorded. The introduction of wooden boxes was followed by a change in sheltering habits. Possums showed a marked preference for the wooden dens either alone or in groups with as many as six possums inhabiting one of the larger boxes at one time. Individual plastic shelters were subsequently used less often.

(iii) Survey possums : Landcare supplied twelve possums from each survey site. When it was feasible, equal numbers of males and females and young or adolescent possums were provided. The survey protocol, to investigate 10 animals per site, allowed for some choice as to which individuals were sampled. As the processing protocol became more complex, the number of possums processed from each sample site decreased to eight or less when other important investigations were under way or when ill health in captive possums (400 series) reduced the number available for processing (Table 3.3).

3.3.2 Co-cultivation : Investigations and findings.

(i) Co-cultivation I : Tissues from 82 possums (series 000 - 900) were co-cultivated with three different marsupial cell lines (Table 3.3). Non-specific deterioration took the form of plaque-like "shrinkage" holes around the perimeter of wells and frequently involved non-inoculated control wells. Neither haemadsorption, completed for 65 possums, nor cytological abnormalities in chamber slides, completed for 61 possums, were detected in monolayers inoculated with the seven tissues from co-cultivation I. There were no cytological changes in monolayer cells adjacent to areas of non-specific deterioration when these phenomena were encountered in chamber slides. A cytopathic phenomenon was differentiated from non-specific deterioration of monolayers on one occasion.

The co-cultivation of a ten fold dilution of a liver homogenate from possum 705 (death in captivity : hepatitis was the main necropsy and histological finding) on PRT-1 cells resulted in a CPE on first passage. Cell retraction and partial detachment from the culture surface gave the appearance of a sub-confluent monolayer. This change was detected over 70% of the culture surface but did not have a focal appearance. A similar CPE was also detected in PRT-1 cells inoculated with kidney homogenate, at the same dilution, from possum 705. In a second passage, monolayer deterioration with a similar but more focal CPE was detected in PRT-1 and PTK2 cells. The standard co-cultivation procedure (liver kidney and lung tissues only) for possum 705 was completed and aliquots from wells showing CPE investigated in dedicated parallel cultures. Although a CPE appeared to be reproducible through two passages, this phenomenon could not be further maintained. Electron microscopic examination of cell culture material (second passage : PTK2 well infected from a PRT-1 well originally inoculated with 705 liver) demonstrated three virus-like particles in the culture supernatant. These particles had an adenovirus or reovirus-like appearance but resolution was insufficient for definite morphological identification (Figure 3.3).

(ii) Co-cultivation II : Twenty six possums were investigated using this procedure. Tonsil chunks (explants) from possum 608 induced a cytopathic effect in PRT-1/2 and PTK2 cells that consisted of focal rounding and detachment of cells around localised

plaques. On second passage, a more localised but similar plaque-like CPE became smaller during the period of passage and in subsequent passages was not detected. No other possum co-cultures, from the second set of tissues investigated, demonstrated evidence for a viral CPE. Neither haemadsorption (20/26) nor cytological changes in chamber slide cultures (20/26) were detected in monolayers inoculated with the six additional tissues from co-cultivation II.

(iii) Explant primary cultures : Six tissues from 26 possums were explanted. Radial fibroblastic cell growth occurred from fragments of all possum tissues but was poor or short lived in lung and lymph node samples. Each of the other tissues explanted (ovary, tonsil, kidney, prostate and salivary gland) formed sizeable cell sheets and monolayers that frequently became independent of the original fragments. The success of individual tissues from different possums varied considerably as did the time it took for primary cell sheets from explants to become maximal (5 - 20 days). Ovary, prostate, kidney and tonsil monolayers were passaged when they appeared healthy and confluent but did not re-establish in six-well multiplates following trypsinisation (1:2 split). Primary cell sheets that did not achieve confluency (large majority) remained stationary for prolonged periods. During this time the cells changed from a predominantly fibroblastic morphology to a predominantly epithelial morphology and became vacuolated. Coverage of the culture surface was reduced as the cells became 'stretched' to stay joined to neighbouring cells and eventually lost contact. These effects were first noticed at the perimeter of the monolayer and eventually progressed to cell rounding and detachment. This common pattern of primary cell deterioration was not differentiated from a viral CPE.

(iv) Explant primary culture passage : In 25 out of 26 possums at least one tissue established a partial monolayer (explant outgrowth) which was maintained and observed for deterioration. In 25 cases outgrowths were passaged a second time (co-cultivated). For the majority of possums (21/26), explant outgrowths were passaged twice and tested by haemadsorption and chamber slide examination. No cytopathic effects were detected. No haemadsorption or abnormal cell morphology (chamber slides) was demonstrated.

Table 3.3 Summary of possum survey : Cultivation and virus detection procedures : Number of possums completed.

POSSUM SERIES	GEOGRAPHICAL ORIGIN	I N V E S T I G A T I O N S									
		CC-I	HA-I	CS-I	CC-II	HA-II	CS-II	EP	EPP	EPP-HA	EPP-CS
000	Hawkes Bay	9	-	-	-	-	-	-	-	-	-
100	Paraparaumu	10	10	9	-	-	-	-	-	-	-
200	Wanganui	10	8	8	-	-	-	-	-	-	-
300	Orongorongo	11	11	11	-	-	-	-	-	-	-
400	Kawau Island	6	6	6	-	-	-	-	-	-	-
500	Banks Peninsula	10	10	10	1	-	-	1	1	1	1
600	Pelorus (Nelson)	8	8	8	7	4	4	7	7	7	7
700	West Coast Region	8	8	5	8	6	6	8	7	7	7
800	Invercargill	6	4	4	6	6	6	6	6	6	6
900	Chatham Island	4	**	**	4	4	4	4	4	**	**
Total possums per investigation		82	65	61	26	20	20	26	25	21	21

CC = Co-cultivation : CS = Chamber slides : HA = Haemadsorption : EP(P) = Explant primary (passage).

CC-I = Co-cultivation of liver, kidney, lung, spleen, lymph node, salivary gland and prostate or ovary. ** = Techniques not completed.

CC-II = Co-cultivation of trigeminal ganglia, lumbar, dorsal root ganglia, brain, buffy coat, tonsil and filtered faeces and/or intestinal contents.

EPP = Passage of stored primary explant cultures : Ovary, kidney, tonsil, salivary gland and prostate.

(v) Miscellaneous possums : In addition to the 82 possums investigated from survey sites, eleven possums were examined from other sources. Five were locally caught, used for specific aspects of technique development, and investigated by co-cultivation and other protocols under way at that time. One possum (RK001) submitted from a research colony at Ruakura with inappetance and an ocular discharge was negative for viruses using the co-cultivation I procedure. However, an oral (glossal) squamous cell carcinoma 15 mm in diameter was identified from this possum. Tissues from three possums from a disease outbreak in Rotorua were passaged through the co-cultivation I and/or explant primary culture procedures. Details of these investigations form part of chapter seven. Two possums that were part of separate projects to cultivate (transmit) adenoviruses and papillomaviruses *in vivo* were investigated using the co-cultivation I protocol at the completion of the transmission trials. No viruses were detected from the 11 possums examined from miscellaneous sources. However, haemadsorption and chamber slide examination was only completed in three cases.

Intestinal contents samples had been collected and stored from a number of possums that were investigated early in the survey period and filtered intestinal contents from 13 of these possums were passaged using the co-cultivation I protocol. This was in addition to the possums that had filtered intestinal contents cultured as part of the co-cultivation II protocol. These cultures were negative for viral CPE, haemadsorption and abnormal cytological appearance of stained monolayers (chamber slides).

A total of 93 possums were investigated by the co-cultivation I procedure and 26 possums investigated more thoroughly using the co-cultivation II and explant primary culture procedures. No sustainable cytopathic effects were reproduced following passage and neither haemadsorption nor abnormal chamber slide cytology was identified in monolayers examined by these techniques. Details are contained in table 3.3.

3.3.3 Primary cells.

Enzymatic digestion - Trypsin : Primary possum kidney cells (PPK) and baby possum kidney cells (BPK), disaggregated by trypsin and seeded at an adequate density ($\geq 2 \times 10^6$ cells/ml), usually formed a predominantly fibroblastic monolayer. The number,

viability and growth rate of cells collected following trypsinisation varied. Fibroblastic morphology of primary cells changed to an epithelial morphology with sub-culture.

Enzymatic digestion - Dispase : Primary PK and BPK monolayers were established following Dispase® digestion overnight with primary cultures confluent 2-3 days later. Monolayers were also established on glass inserts in multiplate wells. Cells plated at 2×10^6 cells/ml following dispase disaggregation grew more quickly than cells digested by trypsin. However, once established by either method, primary cell monolayers appeared and behaved identically.

3.3.4 Establishment of cell lines.

Primary cells from ovary and uterus explants (adult possums) grew out strongly in small flasks and were re-established following trypsinization. These cells were passaged beyond the expected level for primary culture and formed a stock of lower passage possum cells for use in the co-cultivation assay. These cells were passaged more than twenty times with no change of morphology or loss of viability. Possum ovary and uterus cells were differentiated as stock cell lines in liquid nitrogen, but were used interchangeably in the tissue culture survey where provision of the lowest passage (and presumably most sensitive) possum cells was the main objective. Differences between these apparently identical cells were not investigated and although generically referred to as possum reproductive tract cells (PRT-1/2), the actual origin of the possum cells used in most passages was recorded.

3.3.5 Sensitivity of tissue culture for detection of MaHV-1 (positive control).

Where salivary gland tissue and MaHV-1 virus were concurrently added (table 3.2), viral CPE was present in all wells on first passage. All wells inoculated with salivary gland tissue alone had intact monolayers and all wells inoculated with virus alone had detectable CPE. No further passages were undertaken. Kidney, liver, spleen, lung and brain multiplate wells, where tissue at 10^0 had been added, were obscured on first pass. One spleen and one liver well with tissue added at 10^{-1} was also obscured during pass one. All other wells had monolayers where virus was absent and a CPE where virus was present.

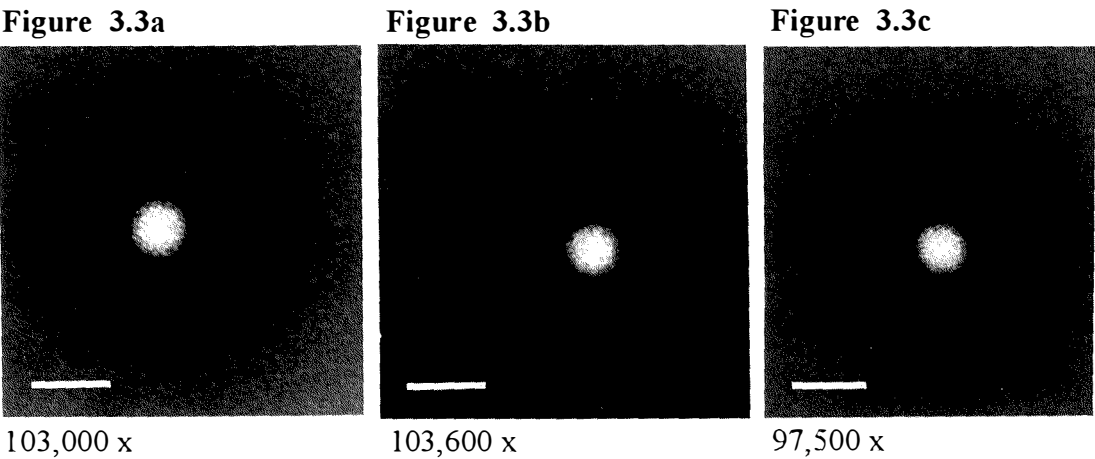
In the second passage, spleen, brain and lung tissue multiplates had viral CPE observed in all wells in which both virus and tissue were added together. Intact monolayers remained in all wells inoculated with tissue alone whereas CPE was present in all wells inoculated with virus alone (titration endpoint beyond the 10^{-5} dilution limit set by multiplate). No further passages were undertaken. Viral CPE was not detected in liver and kidney multiplates where virus had been added with concentrated tissue suspensions (10^0). All other wells which had originally received virus were positive for CPE.

In the third passage (liver and kidney multiplates only) there were no changes. Monolayers remained in all wells that had originally been inoculated with concentrated tissue suspension. Viral CPE was detectable in all wells where tissue had been added at a dilution of 10^{-1} or less and virus concurrently added. Wells inoculated with virus alone contained CPE and wells inoculated with tissue alone retained intact monolayers.

3.3.6 Electron microscopy.

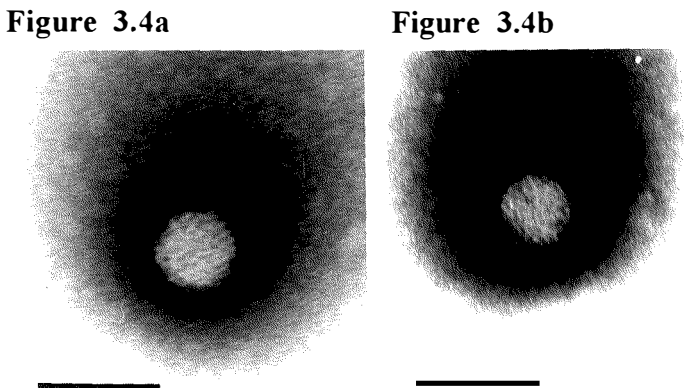
Electron microscopic examination of faeces was performed on 16 possums. Small and large intestinal contents were examined by EM for 28 and 29 possums respectively. A total of 44 different possums had either intestinal contents or faeces screened. Adenoviruses were identified morphologically in the large intestinal contents of three possums from Kawau Island and in the faeces of one possum from Nelson. Typical mammalian adenovirus morphology was observed under high magnification and photographic records confirmed both the physical characteristics and size of the particles (figure 3.4).

Figure 3.3 Negatively stained virus-like particles from possum 705.
Magnification and bar representing 100 nm are supplied for each figure.



Virus-like particles resembling an adenovirus or reovirus were detected by EM from one of the second passages of liver tissue on PTK2 cells. Culture media and monolayer cell debris, following two freezing and thawing cycles, were prepared by negative staining from ultra-centrifuged tissue culture supernatants.

Figure 3.4 Adenovirus particles from possum 406 : Negatively stained x 162,400.
Bar representing 100 nm is supplied for each figure.



Adenovirus particles from large intestinal contents of possum 406. Adenovirus particles were detected in faecal of intestinal contents samples from four survey possums.

3.4 DISCUSSION

Tissue culture methods were developed in order to investigate the viral flora of N.Z. possums caught in different regions. A survey of captive possums was completed and tissues from 93 possums, most of which were healthy at the time of processing, were subjected to virus isolation procedures. Virus isolations were not made and this raised the question of how to most effectively screen a wild population for viral pathogens. Re-evaluation and development of protocols to achieve the most sensitive detection system continued through-out the survey period. This led to a decrease in the number of possums processed per site and an increase in the number and complexity of tissue culture investigations performed per possum. The end result was a comprehensive initial sampling and cultivation protocol which could accommodate additional investigations. The project was ambitious for the resources available and it was often a challenge to prioritise investigations especially where completion of procedures that had been unsuccessful competed for attention with new and untried ideas.

Phenomena resembling viral CPE were detected on two occasions from co-cultivation of liver (CC-I) and tonsil (CC-II) samples. Tonsils (or pharyngeal lymphoid tissues) have a primary defense role against pathogens and many host species have viruses that replicate preferentially and/or remain latent at these sites. Liver tissue filters blood from the gastro-intestinal tract as well as being an important target tissue for many viruses. Further investigation of both these cases is indicated. The one virus-like particle that was detected from culture supernatants following passage of liver tissue from possum 705 resembled an adenovirus. Even though the CPE was not maintained, the detection of this particle was encouraging against the background evidence for a possum adapted adenovirus and molecular techniques to determine whether or not an abortive adenovirus infection was established will become a separate project.

Co-cultivation techniques for virus isolation are routinely used in diagnostic virology (Specter, 1986). Human immunodeficiency virus (HIV), for example, is most successfully isolated following co-cultivation of WBC with mitogen stimulated peripheral blood mononuclear cells (Hirsh and Curran, 1996). Virus induced CPE can occasionally be detected but assay of culture supernatants for antigen or reverse transcriptase activity

reliably indicates the presence of HIV. Co-cultivation techniques have also stood the test of time in research. More than 25 years ago a guinea pig herpesvirus was isolated by co-cultivation of lymphoblasts on guinea pig embryo cultures (Nayak, 1971). Almost identical procedures are currently in use to isolate equid herpesviruses 1, 2 and 5 from peripheral blood leucocytes in this laboratory (J.J. Donald, M. Dunowska, pers. comm.) and many of the basic principles are unchanged from early isolations of equine herpesvirus-2 (Kemeny and Pearson, 1970). Complex co-cultivation protocols have recently isolated a herpesvirus from Kaposi's sarcoma of AIDS patients (Foreman *et al.*, 1997). While specifically applicable for the detection of cell-associated (e.g. beta herpesviruses) or latent viruses (e.g. alpha herpesviruses in sensory nerve ganglia), co-cultivation also detects viruses that are free in body secretions and was therefore considered to be the most appropriate tissue culture method for this survey.

Environmental stressors and/or the administration of exogenous corticosteroids can result in immunosuppression which has been associated with recrudescence of viral shedding. The literature supporting this phenomenon with respect to herpesvirus latency is vast. Dexamethasone treatment of calves resulted in reactivation of viral excretion in nasal secretions of calves previously infected with infectious bovine rhinotracheitis virus (IBRV) (Sheffy and Davies, 1972; Davies and Duncan 1974; Narita *et al.*, 1981). Davies and Duncan (1974) also recovered IBRV from vaginal secretions following corticosteroid treatment and the sacral ganglia of calves were later identified as the site of latency for the venereal strain of bovid herpesvirus-1 (infectious pustular vulvovaginitis virus) using *in situ* hybridisation and auto-radiography (Ackermann and Wyler, 1984). Sciatic neurectomy and decompression involving minimal stimulation to the trigeminal nerve root were sufficient to reactivate latent herpesvirus infections in an animal model and human patients respectively (Walz *et al.*, 1974; Pazin *et al.*, 1978). The breadth of work and number of species in which herpesvirus latency has been investigated is wide with edited veterinary perspectives available in the field (Wittmann *et al.*, 1984). In the current study, corticosteroid administration (in addition to artificial husbandry) was a realistic attempt to increase captivity stress and any associated increases in virus shedding.

Viruses have been detected following the spontaneous deterioration of primary cell cultures, often established for unrelated studies. Adenoviruses gained their name following the spontaneous degeneration of primary explant cultures of human adenoids, first observed by Rowe in 1953 (Horwitz, 1990a). Primary calf testicle cell cultures derived from apparently normal slaughterhouse calves degenerated with a typical adenoviral CPE during routine passaging of non-cytopathic bovine virus diarrhoea virus (Búrki *et al.*, 1978). A combination of explant and CC techniques yielded two equine adenovirus isolations from three cases of cauda equina neuritis (Edington *et al.*, 1984). In this study, explants were attached to flasks prior to the addition of equine foetal kidney cells. When 80% of the radial explant growth had detached from the culture surface (10-14 days), remaining cells were trypsinized and fresh foetal kidney cells added. In this manner seven passages, 10-14 days apart, were performed and isolations made on the fourth and sixth passages. While similar methodology may be applicable to the cultivation of the possum adenovirus, such elaborate protocols were not feasible as part of this survey for logistical reasons.

Adenoviruses achieve latency in intestinal and pharyngeal locations but, in contrast with well characterised herpesvirus examples of latency, the mechanisms are not known (Horwitz, 1996). In a veterinary example, clinical ovine adenovirus infection was reactivated in lambs, following corticosteroid administration, that had recovered from adenovirus infection (Sharp *et al.*, 1976; Pálfi *et al.*, 1982). These early studies did not routinely isolate adenovirus from reactivated infections and failed to fully differentiate viral persistence from latency. However, the use of corticosteroids to assist recrudescence of adenoviral infections was demonstrated and provided further support for the use of this approach in the survey since there was already evidence for the presence of a possum adenovirus (Rice *et al.*, 1991; Rice and Wilks, 1996).

Adenovirus CPE may only be detected after several weeks of passage when first propagated *in vitro* (Edington *et al.*, 1984; Horwitz, 1990b). To maximise the chances of isolating a possum adenovirus there was a compromise between the length of time individual monolayers could be maintained and the number of cultivations that could be attempted. Final cultures were kept for one month in order to detect a presumably

fastidious and slow growing virus.

No viruses were detected by haemadsorption (HA) or chamber slide examination. Viruses haemadsorb or haemagglutinate under widely differing conditions of temperature, buffer composition, pH and source of red blood cells (RBCs). Although a previously used protocol (Horner, 1992) to cover a wide number of virus classes was adopted, a single protocol to detect all possible viruses was not feasible. Monolayers (PTK2 particularly) would detach with excess handling and therefore it was not realistic to use a wider range of different HA conditions. The desire to retain sterility to permit further passaging also influenced the amount of handling that was possible. Chamber slide examination could detect occult virus replication only when a morphological change, such as inclusion body or giant cell formation, was present. Therefore, non-cytopathic viruses, that neither haemadsorbed under the conditions employed nor produced detectable cytological changes in monolayers, for example non-cytopathic bovine viral diarrhoea virus, would have been missed during the tissue culture survey.

The sensitivity of co-cultivation for isolation of MaHV-1 in various tissue samples demonstrated that concentrated inocula derived from liver and kidney tissues prevented detection of virus. The importance of the tissue source and concentration for initial inoculation onto multiplate monolayers was emphasised. Liver and kidney tissues appear not to have been adequately screened and this may have prevented viruses with a distinct tropism from being detected (eg hepatitis viruses). Viruses with a wide variety of target organs, present in many tissues during initial replication or distributed in blood or blood cells, would be likely to have been detected from another tissue source. Prostate tissue was the only "obscuring" sample not tested in the positive control experiment and it remains unknown whether replication of MaHV-1 (monolayer viability) in the presence of concentrated prostate tissue was possible or not. As the inoculum prepared from prostate mincing was physically different from all other tissues it is not possible to extrapolate from the samples that were tested.

The mechanism preventing viral replication at high concentrations of kidney or liver was not determined. Addition of tissue samples prior to addition of virus may have physically

hindered MaHV-1 particles from reaching the monolayer although this mechanism should also have operated for lung and spleen samples. The blood-rich lung and spleen samples differed from liver and kidney samples which, following mincing and elution of tissue components, were more brown than red and did not clot. It appeared that a greater variety of tissue products were released by mincing liver and kidney and that a toxic effect (tissue debris and enzymes affecting target cells) was the most likely mechanism preventing virus replication. Herpesvirus entry into cells occurs following receptor-mediated binding (Roizman and Sears, 1990). Therefore, an alternative mechanism for a toxic effect may have been the damaging of viral envelope glycoproteins and/or cell receptors and the prevention of the virus-host interaction. An experiment was performed to assess cell viability under tissue overburden by removing tissue samples at 1, 3, 5 and 7 days following inoculation (data not presented). It was technically demanding to remove tissue overburden without harming monolayer cells but it appeared that cells remaining beneath spleen samples were viable whereas those beneath liver samples were not.

The production of primary cells for use in virus isolation was fundamental to the development of a sensitive isolation system. Two morphologically identical cell lines, generically referred to as PRT cells, were developed and incorporated into the survey protocol interchangeably. Although it is highly unlikely that both the PRT-1 and PRT-2 cell lines are identical in all respects, it was not possible to assess their degree of permissiveness for unknown viruses. It was argued that for the purposes of virus isolation, cells of the lowest possible passage were likely to provide the most favourable conditions for cultivation of possum viruses and that this advantage outweighed any possible differences that there might have been between the PRT-1 and PRT-2 cells. Production of primary PK cells by the dispase method was developed relatively late in the survey period. Although more work was required to reliably establish these cells as monolayers (this included supply of fresh possum kidneys and the use of small flasks or glass inserts in multiplates) it may have been preferable to substitute primary PKs for either the PK or the PRT-1/2 cell lines. Both PTK2 cells and OPK cells supported the growth of MaHV-1 and were therefore thought to be potentially able to support the replication of possum viruses. These arguments determined the choice and maintenance

of these cell lines for the duration of the survey even though no isolations were made. Dorcopsis wallaby herpesvirus 1, renamed MaHV-2 (Johnson *et al.*, 1985), was first isolated in primary chicken embryo fibroblasts (PCEFs) (Wilks *et al.*, 1981) and the feasibility of using PCEFs in the survey was investigated. As with primary PK cells the extra work to provide fresh tissues, prepare glass inserts for multiplates or small flasks for passage, prevented the use of PCEFs except for special cases. Whatever range of cell lines was to be used in the survey, the decision would need to be justified against finite resources for preparation, passage and storage of cultures during the survey. Other sensitive indicators of viral replication, expensive (including ethically) *in vivo* suckling rodent or egg assays, were omitted for similar reasons.

The choice of cell line is largely determined by the target virus. If there was an undisclosed target virus for the survey it would have been a possum herpesvirus closely followed by a possum adenovirus. Herpesviruses should, in all likelihood, have been cultivable through one of the techniques employed on one of the cell lines employed. On the other hand, adenoviruses are often notoriously difficult to cultivate and the methods employed to grow some of the refractory adenoviruses would be unrealistic to incorporate into a basic screening protocol (Tiemessen and Kidd, 1995).

The production of two new possum cell lines and the development of techniques for cultivation and manipulation of primary possum cells was a successful survey outcome of ongoing benefit to researchers. The survey workload and concurrent research prevented many interesting phenomena from being re-investigated with newer techniques and ideas as these became available. There is considerable scope for further work on the virus-like CPEs identified during this survey. Repetition and extension of the techniques that demonstrated the virus-like phenomena and particles can be attempted from monolayers and fresh tissues appropriately stored at -70 °C. Sophisticated and dedicated cell culture procedures are likely to be required in order to cultivate the possum adenovirus. Increasing expertise with possum cell manipulations, reported above, suggests that this is now a realistic goal. Although it is impossible to estimate the number of possums that were carrying or shedding adenoviruses during the period of survey captivity it remains quite possible that these pathogens passed through the survey

protocols undetected (especially possums from Kawau Island). Since the completion of the EM screening procedures for adenoviruses in samples of faeces and intestinal contents, PCR amplification of adenovirus DNA, from EM positive samples, has provided further confirmation that the identifications were of adenoviruses (Thomson pers. comm.).

Although the tissue culture survey identified relatively few phenomena worth further investigation, the investment of time and effort in protocol development to streamline production and manipulation of possum primary cells was both realistic and necessary. Reliable methods for producing possum cells should support future research efforts in possum virology. It is unknown how many apparently healthy possums would need to be surveyed in order to detect the viral flora of the species and this approach is unlikely to be repeated. However, a range of cultivation techniques for possum viruses has been described and these may be viewed as the basic approach to detection of a virus in samples from possums showing clinical signs of disease.

3.5 SUMMARY

Ninety three possums were investigated for the presence of viruses by co-cultivation. Possums were housed in socially crowded conditions and strategic corticosteroid administration was used to confer additional stress in the anticipation that "latent" virus infection might be detected. Twenty six of the 82 possums were examined in more detail using co-cultivation of additional tissues and the establishment of explant primary cultures which were assessed for spontaneous degeneration and subsequently co-cultivated. Possum tissues were co-cultivated with three marsupial cell lines and three passages of one week duration were performed for all types of possum cell co-culture. All third passage cultures were examined for viruses that did not produce a CPE by haemadsorption (guinea pig, chick and human 'O') and cytological examination of H&E stained monolayers.

No viruses were isolated but a cytopathic effect, apparent for two passages only, and unable to be subsequently maintained, was detected on two occasions. Adenovirus particles were detected in the faeces or intestinal contents of three possums from Kawau Island and one possum from Nelson. Further investigations of these findings are indicated and efforts to develop specialised techniques in order to adapt the possum adenovirus for *in vitro* cultivation are worthwhile. An experiment to test the sensitivity of the survey protocol for viral detection indicated that undiluted liver and kidney tissues may have acted adversely on the cell monolayer, or on any putative viruses and their interaction with cells, to make detection of viruses less likely. This constraint did not apply to other tissues tested.

The tissue culture based survey enabled methods for the detection of possum viruses to be developed. While no isolations were made, a number of interesting phenomena were observed and await further investigation. Specialised primary possum cell cultivation and associated techniques are an investment for future disease investigations and virus isolations. It is anticipated that virus isolation efforts for the next few years will concentrate on possum populations (wild or captive) where a disease entity is visible or be focused towards cultivation of viruses already associated with possums, for example the possum adenovirus.

Chapter 4

WOBBLY POSSUM DISEASE

4.1 INTRODUCTION

Wobbly possum disease (WPD) was first described by Crown Research Institute scientists at AgResearch, Invermay when individual possums, held in a colony for reproductive studies, were observed to have a range of behavioural and locomotor changes (Mackintosh *et al.*, 1995). Early features of WPD included a fine head tremor, wide based stance and tentative investigatory behaviour when still mobile. Anorexia, progressive ataxia, inability to move, apparent blindness and cachexia usually preceded death. The disease was transmitted to healthy possums by intra-peritoneal (IP) injection of suspensions of liver, spleen or brain from affected animals. Recipient possums developed signs typical of the disease. The possums in which WPD was first detected were housed with feral rabbits at the time the disease was first noted and the presence of partially eaten, aborted rabbit foetuses suggested that rabbits may have been the source of an infectious agent (Mackintosh, pers. comm.). During initial transmission studies at Invermay, possums were housed together after the rabbits had been removed and it appeared that the disease spread from possum to possum by direct contact.

Tissues from the clinically affected possums and live feral rabbits from Invermay were supplied to the possum virus research group at Massey University. This offered an opportunity to study further this newly described disease.

The research goals were:

1. To establish transmission of the disease in possums in a closely monitored environment.
2. To describe the range of histological lesions and establish reliable criteria for confirmation of the disease.
3. To determine whether or not rabbits had a role in the disease.

4.2 MATERIALS AND METHODS

4.2.1 Clinical material submitted from Invermay.

Seven live feral rabbits (*Oryctolagus cuniculus*) and tissues from three possums showing clinical signs of WPD infection were received from Invermay. Brain, spleen, liver, kidney, lymph node, blood and bone marrow samples arrived on ice, on two separate occasions, and were processed immediately. Tissues originating from the diseased possums (I116, K0078 and K0096) were disrupted in a Tenbroek tissue grinder and 10% suspensions prepared in MEM containing 1% FBS and 2% PSK. Tissue culture monolayers and recipient possums for inoculation of tissue suspensions were prepared in anticipation of arrival and all submitted tissues were held at 4 °C for the duration of processing. Tissues and tissue suspensions remaining after inoculations were stored at -70 °C.

4.2.2 Experimental animals.

Possums : Possums from the Chatham Islands and from Stewart Island were available for the initial transmission experiments. For later studies, locally caught animals were used. Possums were maintained in individual cages (measuring 50 x 40 x 70 cm) and fed as previously described (3.2.3). Possums were housed in temperature controlled rooms (22 °C) with a 12 hour light/dark cycle. The cages were within 20 - 50 cm of each other but any direct contact between animals was prevented. Separate food and water bowls for each possum were maintained and disinfected with Virkon™ at the end of the experiments.

Rabbits : The feral rabbits supplied from Invermay were housed in laboratory rabbit cages (measuring 27 x 51 x 33 cm) and fed daily on rabbit pellets (Sharpes Grains and Seeds, Auckland) with supplementary fruit and vegetables. Breeding and social interactions were subsequently fostered by a change from individual caging to a group run situation. The rabbits were provided with wooden boxes and plastic dens for shelter, two feeding stations and one watering container inside a 3 m² enclosure. A deep sawdust litter was provided, to absorb excreta and odours, and was cleaned out weekly.

Mice : Conventional near-term pregnant mice (Quackenbush Swiss) were obtained from Massey University Small Animal Production Unit (SAPU) and maintained in individual laboratory cages on commercial rodent rations.

4.2.3 Possum manipulations.

Chemical restraint : Possums were tranquillised with ketamine (40 mg/kg) by intramuscular injection into the caudal thigh region. For deeper sedation, frequently required for juvenile possums or aroused adults, diazepam (2 mg/kg) was administered by the same route.

Possum inoculation : For establishment and passage of WPD at Massey, the intraperitoneal (IP) route of administration was used. Injection sites, immediately forward of the pouch in females and at an equivalent location in males, and blood collection sites were shaved and swabbed with alcohol prior to inoculation or venepuncture.

Blood collection : Pre-inoculation blood samples were obtained from tranquillised possums following preparation of the jugular venepuncture site. Post-inoculation blood samples were obtained by cardiac puncture (19 g x 1½" needle). Possums were placed in dorsal recumbency and the needle directed cranio-dorsally from a point immediately caudal to the last rib and immediately adjacent to the xiphisternum. Large volumes of blood for serology could be collected by cardiocentesis before euthanasia by exsanguination. When recovery was desired, post-inoculation blood samples were collected by jugular or cephalic venepuncture (23 g x ⅝" needle). Whole blood samples for haematology were collected using EDTA as the anticoagulant. Haematological parameters were determined using an automated system (Cobas Minos[®] Vet, ABX Hematologie), total protein estimations by refractometer and differential WBC counts performed manually. Serum protein determinations and electrophoresis were performed at Batchelar Animal Health Laboratories, Palmerston North, N.Z.

4.2.4 Tissues and tissue preparation for possum inoculation.

Liver, spleen and brain tissue suspensions from Invermay possums were processed as described above (4.2.1) and used to challenge recipient possums. In the first transmission

experiment (tissue source = possum I116) one ml of each tissue suspension was pooled (3 ml in total) and inoculated into each of two possums (LT and ST) by the IP route. The second batch of tissues from Invermay (possums K0078 and K0096) were processed to provide a further stock of infectious material. A 30% suspension of pooled liver, spleen and brain tissues from each possum was prepared. The second group of three recipients (possums A, B, and C) each received 10 ml of the suspension by the IP route. Two possums received tissues from either K0078 or K0096 while a third possum received 5 ml of the pooled tissue suspension from both K0078 and K0096. The third group of recipients (possums "Silver" and "Black") were given five mls each of a pooled 20% suspension of liver, spleen and brain tissues from possums LT and ST.

4.2.5 Possum interactions : Cleaning, feeding, observations and recording.

All possum cages were cleaned out daily and possums were observed, handled (when appropriate) and had their feed and water replenished. The timing of this intervention varied on a daily basis to allow for observation of possums at different stages of arousal. Details of appetite, behaviour, interactions with handler and mobility were recorded. Possums were observed closely for early neurological deficits including head bobbing, raised head carriage and a fine amplitude head tremor. Possums were then removed from their cages and assessed for ataxia or degree of immobility (unwillingness or inability to move; apparent blindness). Vision was assessed by avoidance of obstacles and boundaries, reflex blinking when menaced and tracking of objects (pen or finger) moved silently within the visual field.

4.2.6 A "working definition" and scoring system for Wobbly Possum Disease.

Wobbly possum disease was initially (and minimally) defined as the presence of clinical neurological signs and histopathological changes in the central nervous system (CNS) consistent with a nonsuppurative meningo-encephalitis. A more classical presentation of WPD also included typical histological changes in extra-neural tissues. Possums which died before clinical signs were apparent and possums without lesions in the CNS were difficult to categorise.

There were a range of clinical presentations of WPD and variability was encountered both in the number of tissues significantly affected and the degree of histological change consistent with WPD. An objective method of collating clinical and histological information was required and a weighted scoring system was devised to give a numerical cut off for WPD. Clinical signs and histological changes, separated into two equal categories, were tabulated to take advantage of as many observations as possible. These categories were further sub-divided into five clinical and five histological sections with a maximum of six points that could be allocated for each category and a combined maximum total of 12 (table 4.1 and 4.2).

Table 4.1 Numerical definition of WPD : Clinical indices.

CLINICAL SIGNS				
Ataxia	Immobility	Δ Behaviour	Appetite↓	20% Wt ↓
3 (2)*	(1)*	1	1	1

The six points for clinical signs of WPD were weighted for ataxia (3 points) with one point each awarded for an observable behavioural change, appetite decrease and weight loss of greater than 20%. One common presentation of WPD was an almost complete lack of movement or exploration when released. Possums presenting in this manner (*) were scored with two points for ataxia and a single point to note their immobility.

Table 4.2 Numerical definition of WPD : Histological indices.

HISTOLOGICAL SIGNS				
CNS	Liver	Kidney	Heart	Other
2	1	1	1	1

The six potential points for histological WPD were weighted for typical CNS lesions of meningitis and/or encephalitis (2 points). Lymphocyte accumulations around blood vessels in the liver or kidney, multifocal interstitial nephritis or any abnormal focus of lymphocytes and plasma cells in the myocardium gained one point each. The sixth potential point was for the presence of peri-vascular cuffing or an accumulation of lymphocytes and plasma cells in any other tissue. Scores were collated and the

cumulative totals compared against criteria set out in table 4.3. Scores for possums infected or exposed to WPD during initial trials are tabulated (table 4.9) and this scoring system was used to score possums in the 'Routes of Transmission' and 'Rotorua Syndrome' chapters (chapters 6 and 7 respectively).

Table 4.3 Wobbly possum disease status : cumulative numerical criteria.

CUMULATIVE SCORE	STATUS
RANGE	
0 - 4	Insufficient evidence for WPD
≥ 5	Wobbly possum disease

The minimum definition of WPD (4.2.6) required the cut off value for numerical diagnosis of WPD to be greater than or equal to five.

4.2.7 Euthanasia followed clinical assessment of WPD.

Except where death intervened, possums were allowed to progress to a stage where neurological signs of WPD were unambiguous. This was usually accompanied by a significant decrease in appetite, wasting and a loss of interest in the immediate environment. The decision to kill possums was based on confirmation of the clinical disease by two veterinarians and, for welfare reasons, was done as soon as possums were clearly ataxic and showed no interest in food, handler or environment. Following sedation and blood collection possums were euthanased by intra-cardiac, jugular or cephalic injection with 1 ml of sodium pentobarbitone or by exanguination following cardiocentesis under sedation. Control possums were held for three to four weeks following the euthanasia (or death) of possums that were challenged with WPD.

4.2.8 Post-mortem examination and sample collection.

Possum carcasses were assessed for muscle bulk and overall condition by palpation, inspected for external lesions and weighed. The abdominal cavity was opened following preparation as previously described (3.4). A careful inspection of the abdominal and thoracic cavity was performed and fresh spleen and liver samples were aseptically collected for further passage. A range of tissues were collected into neutral buffered

formalin for histology. The cranium was prised open following encircling, tangential saw cuts, the dura mater reflected from the cerebrum and a longitudinal midline incision made from the olfactory bulbs to the medulla. Starting cranially, one half of the brain was freed from its ventral attachments and collected fresh into a sterile container for further passage as required. The second half of the brain was removed and preserved in formalin for histological assessment. Spinal cord segments were collected from the cervical and lumbar regions on a number of occasions and fixed in formalin. All formalin fixed tissues were processed for histological examination and stained with haematoxylin and eosin. Kidney sections were stained using a modified Warthin and Starry method (silver staining) for the detection of spirochaetes (Warthin and Starry, 1920) and systematically examined for leptospiral organisms.

4.2.9 Size of the infectious agent.

To determine whether the agent of WPD was consistent in size with a virus, a presumably infectious tissue inoculum was fractionated and filtered. Three inocula were kindly prepared by Dr Joseph O'Keefe (AgResearch Wallaceville) from the spleen of possum 1E, which had been challenged with infectious tissues from original Invermay possum I116 and confirmed to have WPD, and transported to Massey at 4 °C. Briefly, the spleen was removed, minced with scalpel blades and extruded through a 50 ml syringe. Minimum essential media (MEM) was added and the disrupted spleen shaken to prepare a 10% suspension. Before the cells had settled an aliquot of 13.5 ml was removed and 1.5 ml of dimethyl sulphoxide (DMSO) added to prepare the **homogenate**. The remaining suspension was centrifuged at 700 x g for 10 minutes and the cell free **supernatant** was collected. A portion of the supernatant was collected and passed through a 0.22 µm filter to produce the **filtrate**.

Three possums were assigned to each of three treatment groups (filtrate, supernatant and homogenate). Two possums in each group received 5 ml of the inoculum by the IP route while the third possum was left as an uninoculated sentinel control. Each possum was placed in a separate cage with the uninoculated control possum placed between two inoculated possums. The three treatment groups were kept in separate rooms and doors were kept closed whenever possible. Feed supplies, sawdust bedding and feeding and cleaning implements were maintained for each treatment group but within each room

these implements were used to clean out and feed both the inoculated and control animals. Access to possums was restricted to three personnel who wore clean gumboots, autoclaved overalls and disposable gloves for possum handling procedures. A strict order for attending the animals was maintained from filtrate to supernatant to homogenate groups. Before entry and after each cycle of attending to the three groups of possums, boots were washed in a Virkon™ footbath and overalls bagged and sent for autoclaving. Gloves were changed between each possum group.

4.2.10 Assessment of rabbits.

After two months of group housing the rabbits were killed. Serum samples were collected from sedated rabbits prior to euthanasia using techniques that have been described for possums (4.2.3) and stored at -20 °C. A careful post-mortem examination was conducted and tissues collected into formalin for subsequent histological examination. Reference slides for the detection of *Encephalitozoan cuniculi* (*Nosema*) organisms, stained using haematoxylin and eosin, were kindly supplied by Professor B.W. Manktelow.

Fresh tissues for storage at -70 °C were collected from all rabbits and fresh uterine tissue and the early concepta from a pregnant doe were homogenised together with fresh rabbit liver, spleen and brain to provide a 20% pooled rabbit tissue suspension. This suspension was centrifuged at 2700 x g for 30 minutes and two possums (602, 603) were each given 10 ml of the supernatant by intra-peritoneal injection. Whole blood from two rabbits was pooled and 10 ml injected intra-peritoneally into a further two possums (600, 601).

4.3 RESULTS

4.3.1 Establishment of WPD at Massey University.

Possums (ST, LT, A, B and C) inoculated with infectious tissues supplied from Invermay developed WPD. Two further possums ("Silver" and "Black"), inoculated with tissues from possums LT and ST, subsequently developed clinical WPD which was histologically confirmed (Table 4.4 Table 4.9). All seven possums developed varying degrees of lethargy and ataxia except possum "Silver" which died following a period of depression, wasting and apparent inability to move that did not involve observable inco-ordination.

All seven possums were in poor condition when killed. All seven possums developed significant nonsuppurative meningo-encephalitis and a range of WPD-associated lesions in extra-neural tissues. Fresh liver, spleen and brain tissue from all possums in table 4.4 was stored at -70 °C and used to prepare a standard pool of infectious tissues for subsequent transmission studies. The clinical signs, haematological changes, necropsy findings and histopathology associated with WPD are presented below (4.3.3, 4.3.4, 4.3.5, 4.3.6).

Table 4.4 Details of early passage experiments.

Possum ID	Inoculum details and source	Passage level	W P D signs (days pi)		W P D Score
			behaviour	ataxia	
ST	I116. 3 ml IP. 10% brain, liver and spleen pooled.	1	7 days	11 days	9
LT		1	7 days	14 days	9
A	K78 + K96. 10 ml IP. 10% brain, liver and spleen pooled.	1	9 days	12 days	9
B		1	8 days	21 days	10
C		1	aggressive	23 days	11
Black	LT + ST. 5 ml IP. 10% brain, liver and spleen pooled	2	nr ¹	14 days	9
Silver		2	13 days	(14 days) ²	9

1. nr = not recorded. An obvious change of behaviour preceding ataxia was not detected

2. Possum "Silver" became moribund and died without showing obvious ataxia

4.3.2 Size of the infectious agent (filtration experiment).

All possums which received homogenate, supernatant and filtrate preparations became ataxic and were confirmed to have WPD. No control animals had clinical signs or histological changes associated with WPD (Table 4.5, Table 4.9)

Table 4.5 Filtration trial possum WPD scores.

ROUTE OF INOCULATION	TYPE OF INOCULATION	INCUBATION (days to ataxia)	WPD	SCORE
Intra-peritoneal	Homogenate	13, 24	2 / 2	11, 9
Intra-peritoneal	Supernatant	15, 20	2 / 2	10, 10
Intra-peritoneal	Filtrate	19, 19	2 / 2	10, 9
Non-contact control (no inoculation)			0 / 3	0, 0, 0

4.3.3 Clinical signs of Wobbly Possum Disease.

The description of the clinical, necropsy (4.3.5) and histological changes (4.3.6) seen in WPD incorporates observations made on all possums inoculated with the Invermay strain of the agent and tissue derivatives following *in vivo* passage. Therefore, some of the behaviours described below and summary statistics were generated by the inclusion of possums involved in the transmission trials described in chapter six. The mean time and range of times to the onset of behavioural changes and ataxia is presented in table 4.6.

Table 4.6 Onset of clinical signs of WPD.

TOTAL NUMBER OF POSSUMS	MEAN TIME TO BEHAVIOUR CHANGE - days	MEAN TIME TO ATAXIA - days
45 exposed	10.1 range 5 - 20 (N = 33)*	16.5 range 11 - 25 (N = 38)*
12 controls	Normal captive behaviour	No controls became ataxic.

* Possums naturally exposed to WPD were not included and records for all possums were not available.

The first signs of disease included loss of appetite, a decrease in arousal at times of interaction with humans for feeding and cleaning (animals could frequently be handled and were easy to move for cleaning purposes) and a more general loss of interest in the environment when compared to controls. There was significant variation between

inoculated animals, assessed qualitatively for the duration of the study period, with behavioural changes ranging from timidity to extreme overt aggression.

Mild behavioural changes were frequently observed during the first week after inoculation. Possums released during this stage would usually explore the environment more slowly than controls unless provoked. Possums would occasionally bump into walls if running away but usually avoided collisions and were able to climb during this period. Raised head carriage and fine amplitude head tremors, as described by Invermay scientists with the original cases, were confirmed in a minority of cases. Possums with WPD would neither consistently follow visual stimuli nor react to menacing. Normal possums were unreliable when menaced but would generally follow objects moved in their field of vision when first presented.

In the second week following inoculation, a decrease in appetite and spontaneous investigatory behaviour was usually observed. Exploratory behaviour during this period was reduced or absent with ambulatory possums displaying a slow, staggering or rolling gait and a wide based stance.

During the final stage of the clinical disease, possums were completely unable or unwilling to move by themselves even when pushed by an investigator. A variable head tremor was apparent in a few animals at this stage if they were stimulated by the cleaning and handling procedure (tremors of any description were rarely observed as spontaneous events). Righting reflexes were retained and all affected possums maintained an upright, tightly hunched posture with heads tucked away from light whilst resting. Less commonly, stimulation would precipitate a convulsive episode of short duration. These episodes resembled a petit mal seizure but were never observed as spontaneous events. A progressive anorexia, varying in severity, was observed in most possums although many affected animals continued to eat small amounts of food (especially fresh fruit or vegetables) up to the time of euthanasia. Frank blood was occasionally detected in faeces, often in association with mucus.

4.3.4 Haematological and biochemical changes associated with WPD.

Blood samples from three affected possums, collected 32 - 43 days following inoculation, and two normal possums were subject to serum protein electrophoresis. These data are presented as raw and summary statistics (table 4.7).

Table 4.7 Serum proteins measured by electrophoresis.

Serum Proteins (g / litre)	WPD + ve (n=3)	WPD - ve (n=2)
Total serum protein (g / litre)	80 ¹ [77.2, 79.7, 82.7] ³	49 [46.3, 52.0]
Albumin (g / litre)	31.3 (39.1) ² [29.5, 29.6, 34.9]	32.5 (66.3) [31.7, 33.2]
α Globulin (g / litre)	14.14 (17.7) [8.95, 10.7, 22.7]	6.07 (12.25) [4.8, 7.3]
β Globulin (g / litre)	20.3 (25.4) [18.1, 21.3, 21.5]	7.63 (15.3) [5.4, 9.9]
γ Globulin (g / litre)	14.2 (18) [5.9, 15.5, 21.1]	2.97 (6) [2.9, 3.0]
Albumin : globulin ratio	0.66 [0.56, 0.59, 0.83]	2 [1.6, 2.5]

1. First figure in box represents the mean value (g / litre).
2. Figures in () parentheses represent the mean value as a percentage of total protein.
3. Figures in [] parentheses represent raw scores (g / litre) and indicate the range of values.

Too few observations were available for meaningful statistical comparison. Albumin values were similar in affected and non-affected possums. The increase in total protein was due to substantial and mostly consistent increases in globulins. In two of the affected possums, γ globulins accounted for greater than 20% of the total serum protein (compared to 6% for normal possums). In the third affected possum, measured six weeks after inoculation, increased α and β globulins accounted for a greater proportion of the total globulin increase than γ globulins, which were within previously published reference ranges (Fitzgerald *et al.*, 1981).

Haematology : Pre-inoculation and post-inoculation blood samples from eight possums were compared. Sample means for the pre-inoculation and post-inoculation groups are presented below (Table 4.8). Unpaired two tailed *t* - tests and the Mann Whitney test (non-parametric, rank sum test) were used to compare the affected and control groups of possums (GraphPad PrismTM version 2.01, GraphPad Software, Inc. San Diego).

Table 4.8

Haematological data for eight possums before and after inoculation with WPD.

PARAMETER	Pre-inoculation	Post-inoculation
Total Protein (plasma) ***	68.5 ² [60 - 75]	84.0 [80 - 89] (6) ¹
Haematocrit ***	0.38 [35 -40]	0.26 [0.21 - 0.33]
RBC ³ (x 10 ¹² / litre) ***	5.9 [5.23 - 6.44]	4.2 [3.52 -4.96]
Haemoglobin (g / l) ***	119 [111 - 128]	87 [65 - 108]
MCV (femtolitres)	65.7 [63 - 68] (7)	64.5 [60 - 73]
MCHC (g / l) *	312.3 [300 - 322] (7)	324.1 [309 - 341]
nRBC ⁴ / 100 WBC ⁵ **	9.7 [0 - 23] (3)	38 [9 - 82]
WBC (x 10 ⁹ / litre)	5.25 [3.6 - 6.74]	5.79 [2.9 - 8.14]
Neutrophils (x 10 ⁹ / litre)	2.48 [1.47 - 4.25]	2.53 [1.36 - 3.41]
Lymphocytes (x 10 ⁹ / litre)	2.41 [1.19 - 3.14]	1.81 [0.58 - 2.52]
Atypical lymphocytes (AL) (x 10 ⁹ / litre)*	0.06 [0.0 - 0.45]	0.98 [0.42 - 2.06]
Monocytes (x 10 ⁹ / litre)*	0.21 [0.05 - 0.4]	0.43 [0.21 - 0.77]
AL + Monocytes (x 10 ⁹ / litre) ***	0.27 [0.05 - 0.71]	1.41 [1.02 - 2.5]
Eosinophils (x 10 ⁹ / litre) *	0.124 [0.0 - 0.4]	0.014 [0.0 - 0.07]
Basophils (x 10 ⁹ / litre)	0.02 [0.0 - 0.13] (1)	0.05 [0.0 - 0.14] (5)

*** P < 0.001 ** P<0.01 * P < 0.05 Statistically significant 1. () Number of observations

2. Mean value and [Range of values] 3. RBC = Red blood cell 4. nRBC = Nucleated RBC

5. WBC = White blood cells, corrected for presence of nucleated RBCs.

Affected possums developed an anaemia based on haematocrit, RBC and haemoglobin concentration differences between groups (significant at the 0.1% level). There was no clinical indication of this process. The MCV of affected possums was not significantly different from control possums but the MCHC was mildly increased (significant at the 5% level). Total serum protein concentration was elevated and nucleated RBCs were more frequently detected in affected possums (significant at the 0.1% and 1% level respectively).

No differences in mean WBC totals were demonstrated between groups. However, WBC values were more variable (standard deviation 2.09 compared to 1.13 for normal

possums) for affected possums. The mean number of lymphocytes in affected possums was lower than in control possums. However, lymphocyte values were highly variable and no significant difference was statistically determined. Atypical lymphocytes were recorded in all of the affected possums but only once in control possums (significant at 0.1% level). Monocytes were more numerous in affected possums and eosinophils were detected more frequently in control possums than in affected possums (both significant at 5% level). Basophils were detected more frequently in affected possums but there were too few observations for meaningful statistical inference.

The morphology of the red cells was subjectively evaluated. In contrast with the regular morphology of red cells in control possums, affected possums displayed mild to moderate anisocytosis and mild polychromasia. Supravital staining techniques for reticulocytes were not performed. Abnormal red cell forms that were frequently detected in affected possums included dacrocytes, ovalocytes (+ "match stick" forms), schistocytes and occasional codocytes. Blister cells and a range of subsequent changes leading to keratocytes (horn cells) were detected in affected possums. Acanthocytes were occasionally observed in affected possums. Echinocytes were detected in both groups of possums. Platelet morphology was not specifically evaluated.

4.3.5 Necropsy findings.

There were few gross changes in the affected possums and no consistent gross lesions at necropsy. Three adult possums and two juveniles that died were dehydrated and in poor body condition (loss of abdominal fat reserves and muscle bulk). Of the possums that were euthanased, there was a variable degree of wasting as assessed by loss of abdominal fat reserves and skeletal muscle bulk (pre- and post-inoculation bodyweight records were not always available). Three possums that had been given a tissue suspension (IP route) during early transmission studies had abscesses in the abdominal cavity but no cases of generalised peritonitis were observed. Gastro-intestinal tracts, the caecum in particular, of inappetant, affected possums were relatively empty in contrast to control possums. Occasionally a clinically affected possum showed paleness of the liver but no other abnormalities were observed grossly. In several of the control animals and particularly in the young and adolescent controls, significant weight gain and

deposition of abdominal fat was observed. Pale, fatty livers were often seen in these animals.

4.3.6 Histological changes associated with WPD.

Consistent histological changes were observed in almost all adult possums identified with clinical signs of WPD. The severity of the lesions varied between animals and tissues within an animal. The predominant lesion was a nonsuppurative inflammation centred around blood vessels in many organs. Veins were more often involved than arteries and were cuffed with mononuclear cells. Lymphocytes dominated the cuffs around blood vessels with plasma cells the next most common cell type. Macrophages were observed occasionally in hepatic and renal lesions and nucleated RBCs were frequently seen in hepatic sinusoids.

Central nervous system : In neurological tissues, light to moderately cuffed blood vessels were found in association with a diffuse increase in the cellularity of the meninges of the brain (figures 4.1 and 4.2). The venous bias of inflammatory cells was evident in the meninges. Large atypical lymphocytes and occasional binucleate forms were identified in the meningeal micro-circulation. A nonsuppurative encephalitis exclusively centred on blood vessels and composed of lymphocytes and plasma cells usually accompanied meningeal lesions and was detectable in both grey and white matter (figures 4.3 and 4.4).

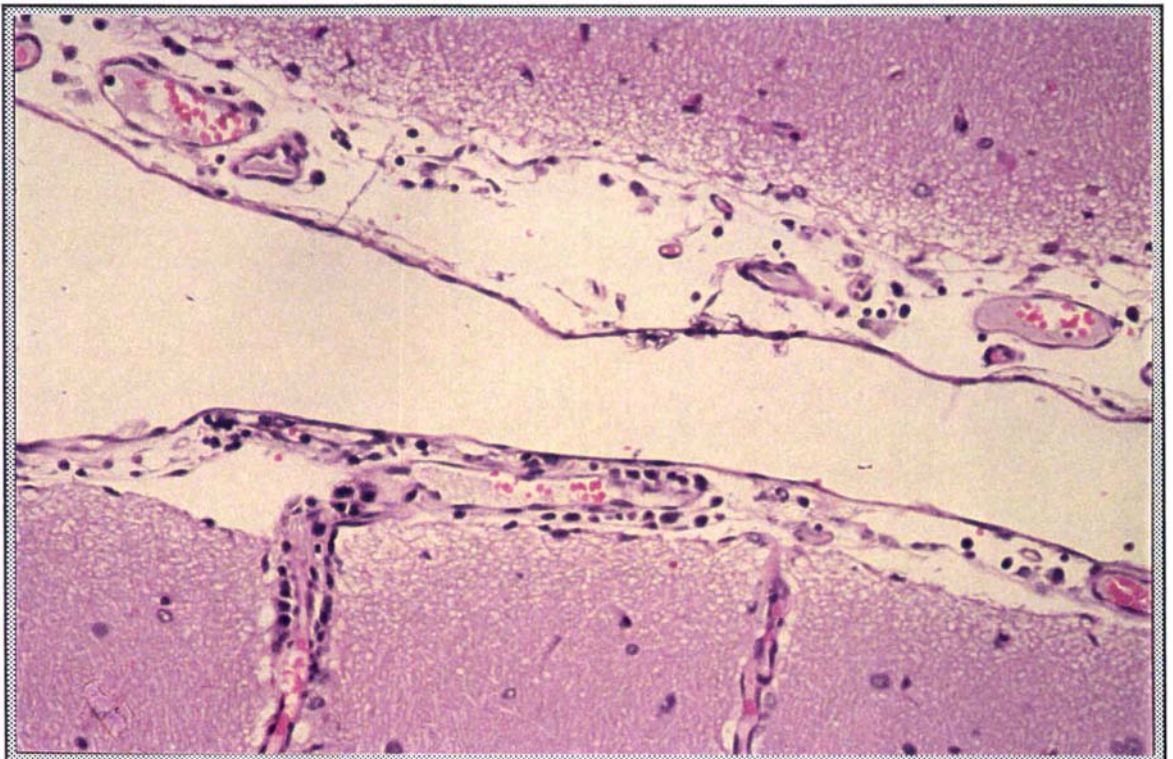
In a small proportion of possums, lesions in the cervical and lumbar spinal cord were similar to the changes observed in the brain. These took the form of significant plasma cell and lymphocyte aggregations around blood vessels in the meninges, grey matter and white matter. However, in the majority of possums affected with WPD the only detectable lesion in the spinal cord was a very mild, diffuse, nonsuppurative meningitis.

Figure 4.1 Section of meninges from possum 3437; H & E stain (x 215).



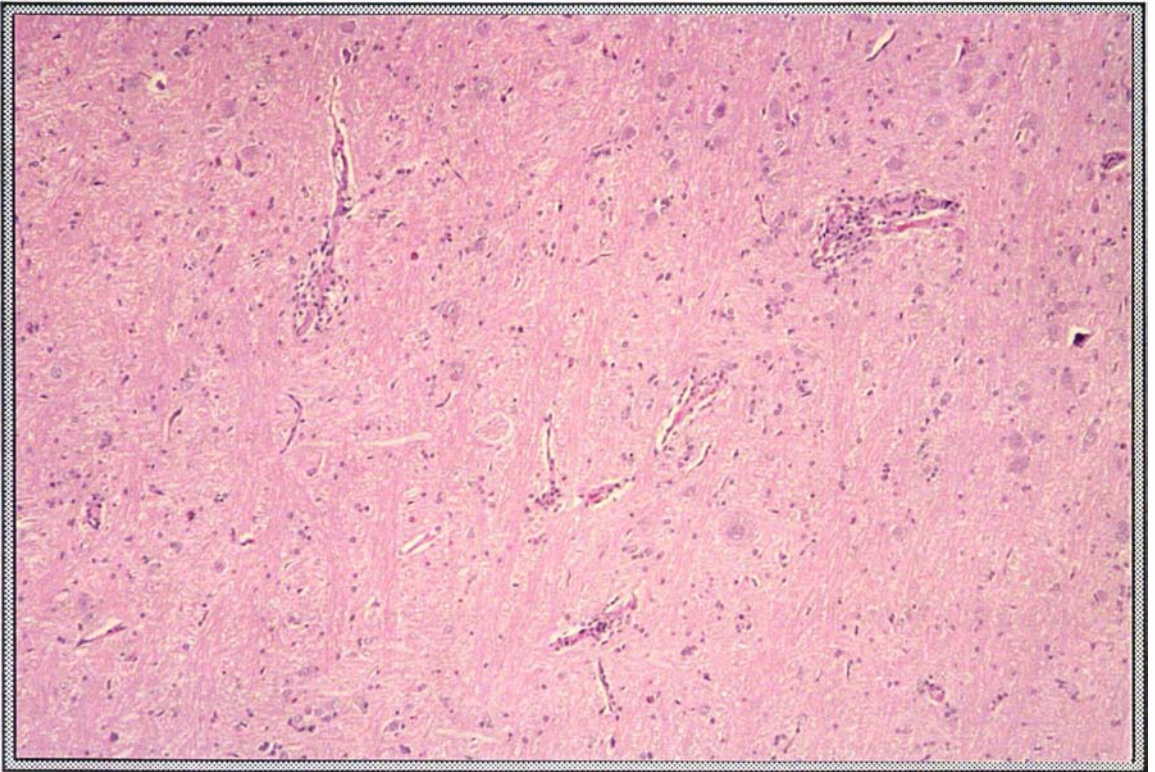
Nonsuppurative meningitis; mononuclear inflammatory cells were more commonly associated with venules than arterioles.

Figure 4.2 Adjacent sections of meninges from possum 3437; H & E stain (x 215).



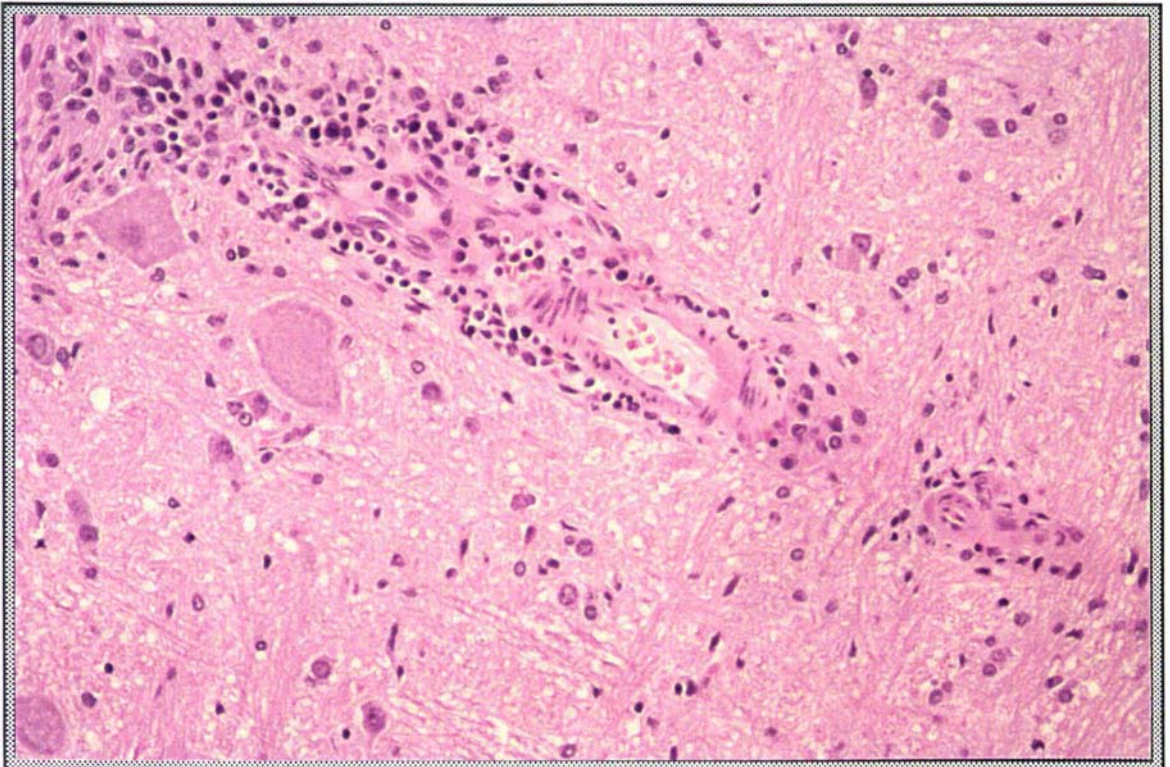
There is a diffuse increase in the number of mononuclear inflammatory cells and these cell types are associated with vessels in the meninges and adjacent vessels in the brain.

Figure 4.3 Section of brain stem from possum 668; H & E stain (x 86).



Nonsuppurative encephalitis; mononuclear inflammatory cells were almost exclusively associated with blood vessels.

Figure 4.4 Section of brain from possum 'black'; H & E stain (x 215).



Mononuclear inflammatory cells and microglial cells were identified in cuffs surrounding blood vessels.

Liver : Lymphocytes were seen in perivascular locations in the liver of normal (wild caught) possums and in control possums and have been reported as an incidental finding in this species (Cooke, pers. comm.). Hepatic, perivascular lymphocyte accumulations in possums with WPD were more pronounced than this and involved both central veins and portal vessels. On many occasions there were foci of inflammatory cells in the liver parenchyma as well as a diffuse increase in the number of cells, including nucleated RBCs, in sinusoids (figure 4.5 and 4.6).

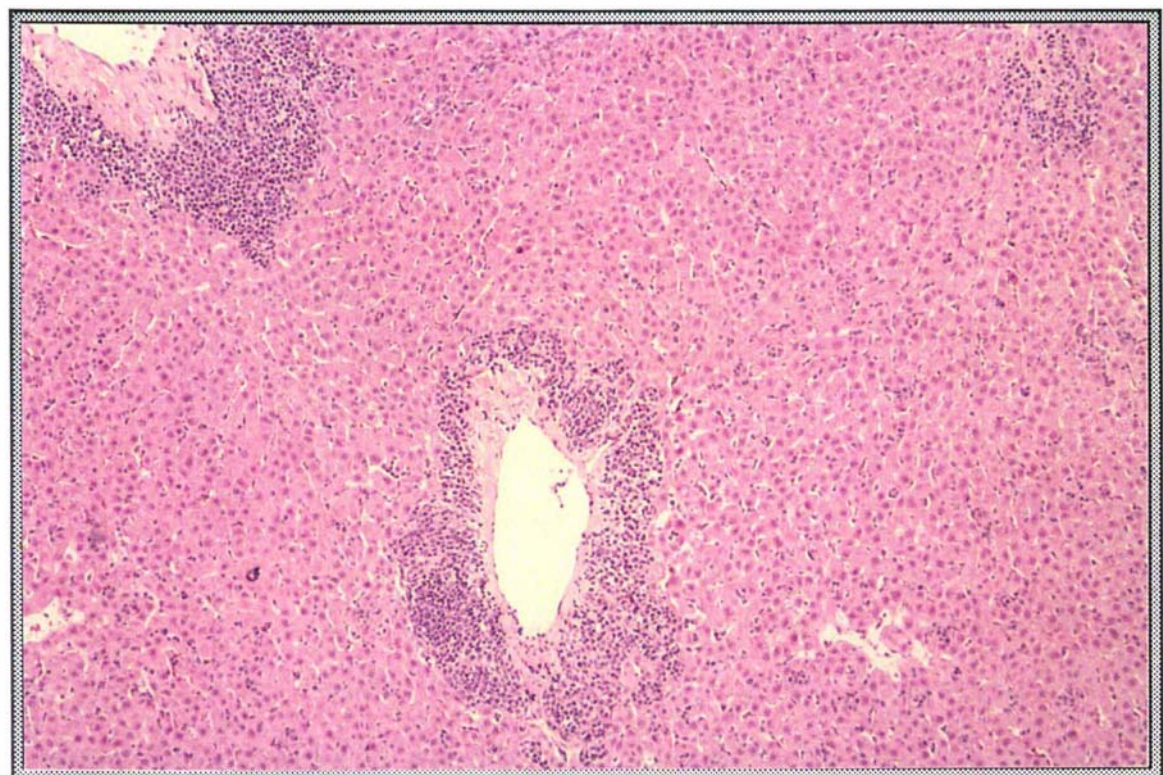
Lymphocyte and plasma cell cuffs around the larger hepatic blood vessels in WPD could usually be differentiated from small lymphoid aggregations seen surrounding such vessels of normal possums by the lack of uniformity of the mononuclear cell population in WPD possums, the greater bulk and distribution of inflammatory cells and the tendency to involve small blood vessels and intra-parenchymal sites. In WPD, mononuclear cell cuffs usually occurred closer to the lumen of the vessels and appeared to protrude into the vessel lumen (figure 4.5 and 4.6).

Kidney: The predominant lesion in the cortical and subcapsular areas in particular was interstitial nephritis (figure 4.7). Lymphocytes and plasma cells frequently surrounded glomeruli but these structures did not appear to be the focus of the lesions. Larger blood vessels (veins) in the cortico-medullary area (occasionally more widespread) were frequently infiltrated by lymphoid aggregations. At their most characteristic, these lesions were an even swelling of the intima, comprised of lymphocytes and plasma cells, that distorted the cross section of the lumen (figure 4.8).

No leptospire were detected in silver stained kidney sections from eight possums.

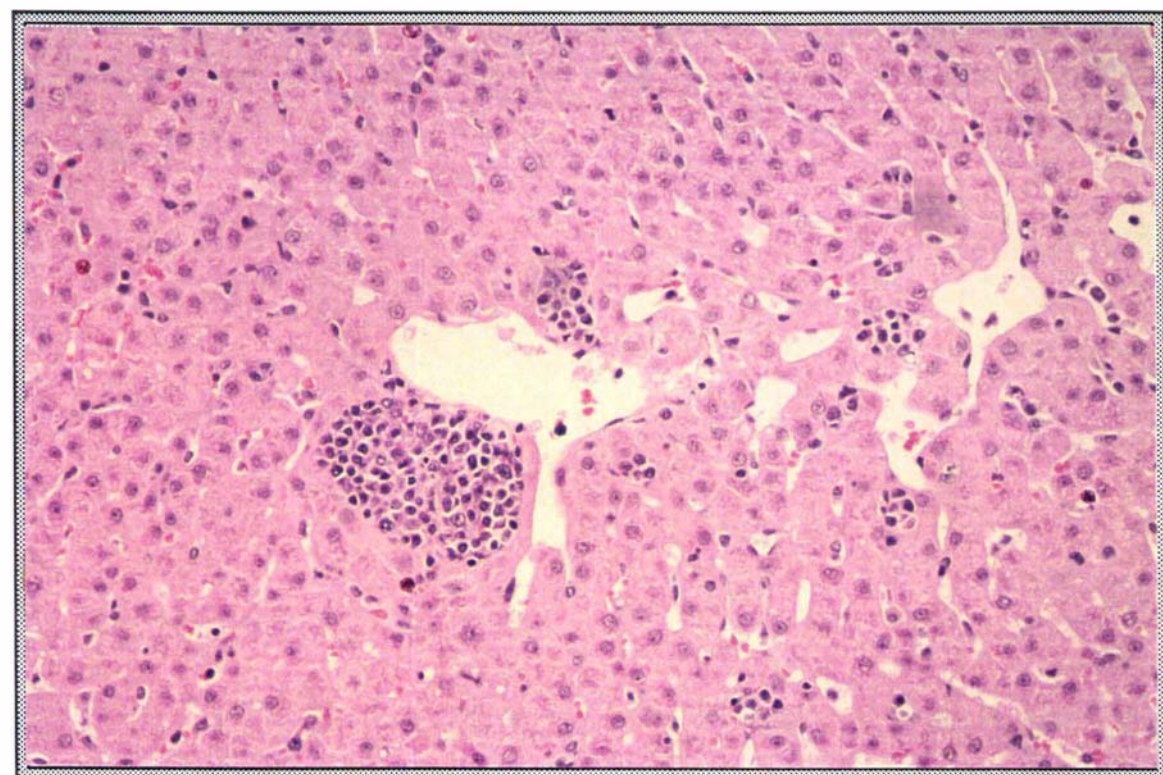
Other tissues: A similar degree of perivascular, mononuclear cell cuffing was sometimes observed around submucosal veins in the urinary bladder (figure 4.9), myocardium (figure 4.10) and adjacent to Schlemm's canal in the eye but in these tissues lymphoid aggregations were also independent of blood vessels. Lymphoid cells were observed beneath the epicardium and endocardium, occupying areas of connective tissue adjacent to blood vessels and as small mononuclear cell infiltrations between muscle fibres. Myocardial lesions were invariably mild in the possums in which these were present.

Figure 4.5 Section of liver from possum 668; H & E stain (x 86).



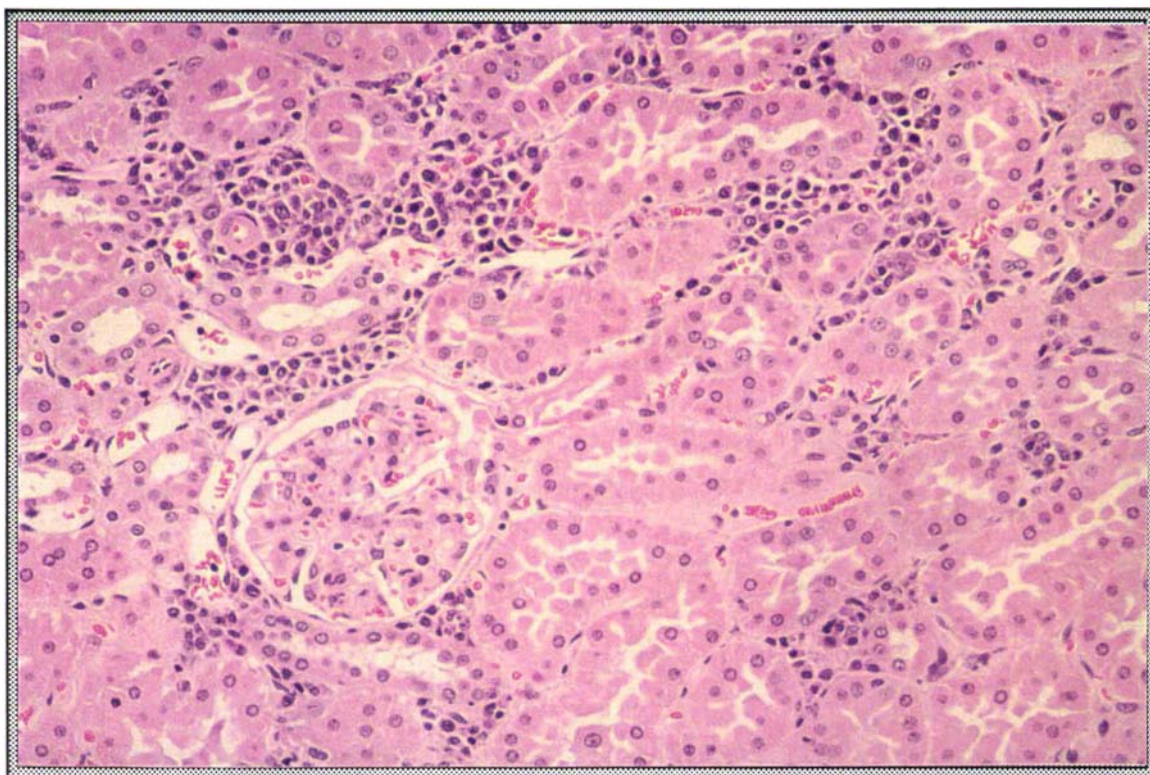
Perivascular accumulations of mononuclear inflammatory cells surrounded the majority of hepatic blood vessels and smaller foci of the same cell types were also observed in the liver parenchyma.

Figure 4.6 Section of liver from possum 667; H & E stain (x 215).



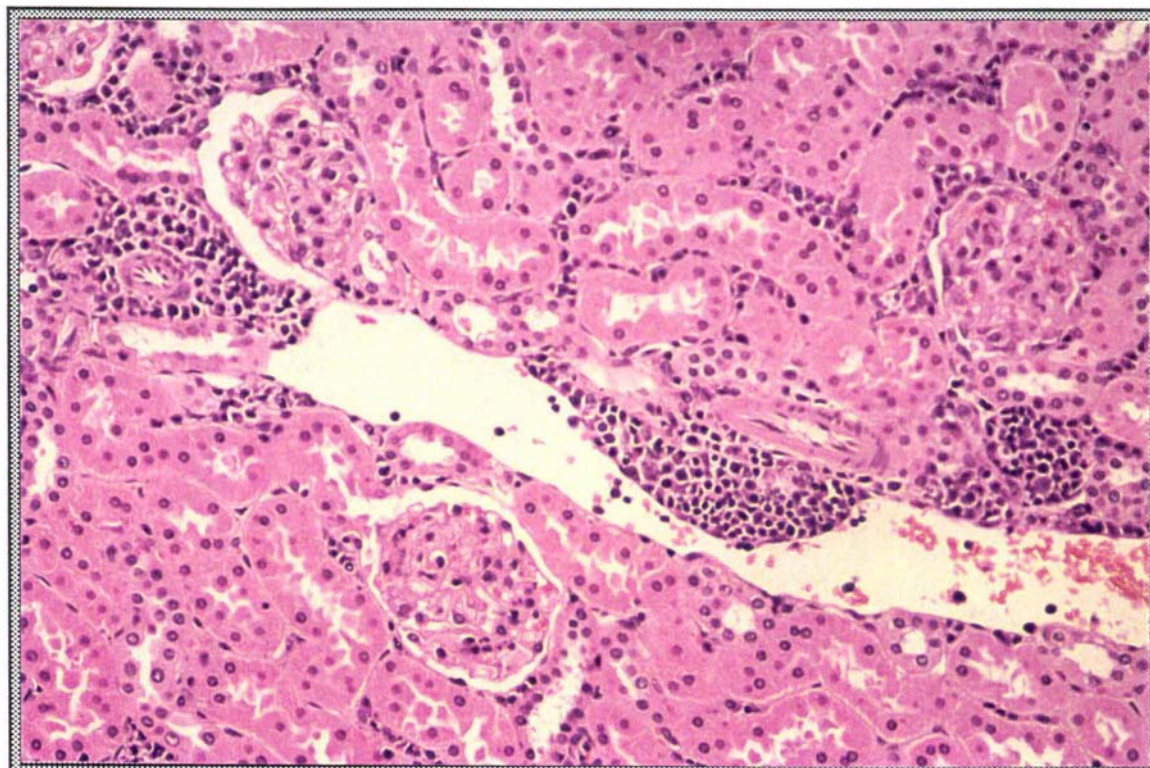
Mononuclear inflammatory cells are closely adjacent to, and associated with distortion of, smaller hepatic blood vessels.

Figure 4.7 Section of kidney from possum 667; H & E stain (x 215).



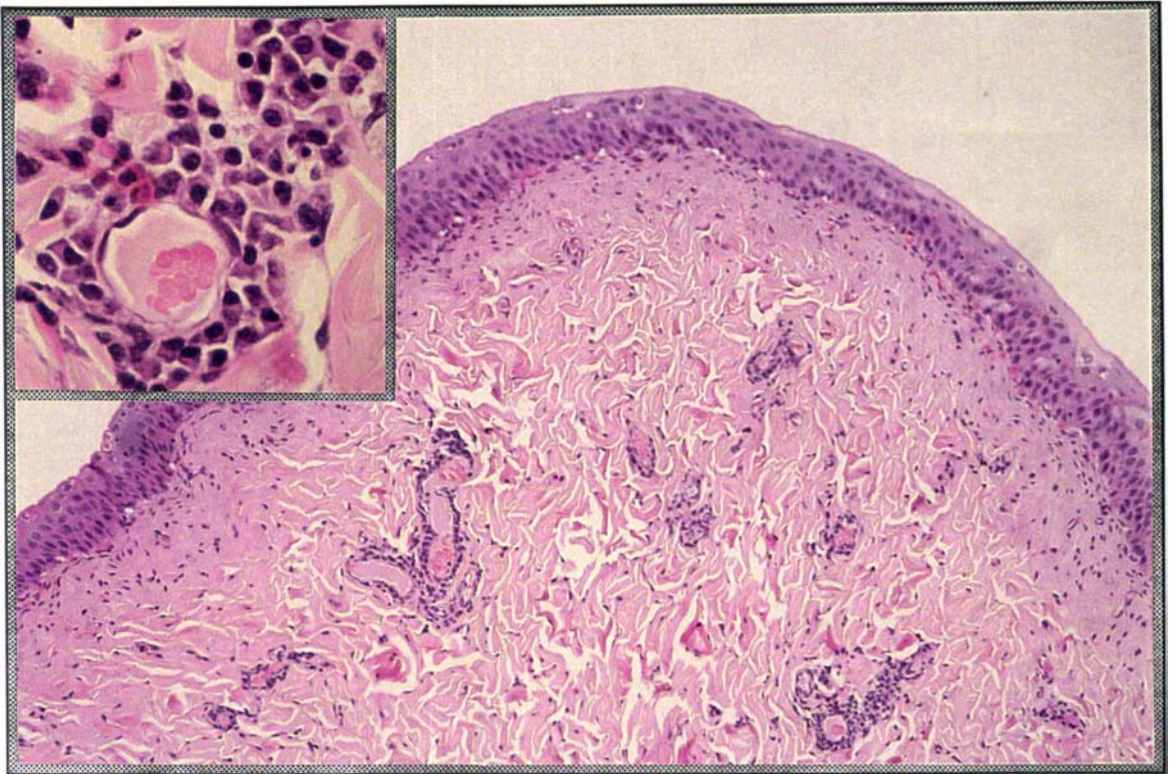
Foci of mononuclear inflammatory cells (predominantly lymphocytes and plasma cells) are seen both associated with glomeruli and interstitially.

Figure 4.8 Section of kidney from possum C ; H & E stain (x 215).



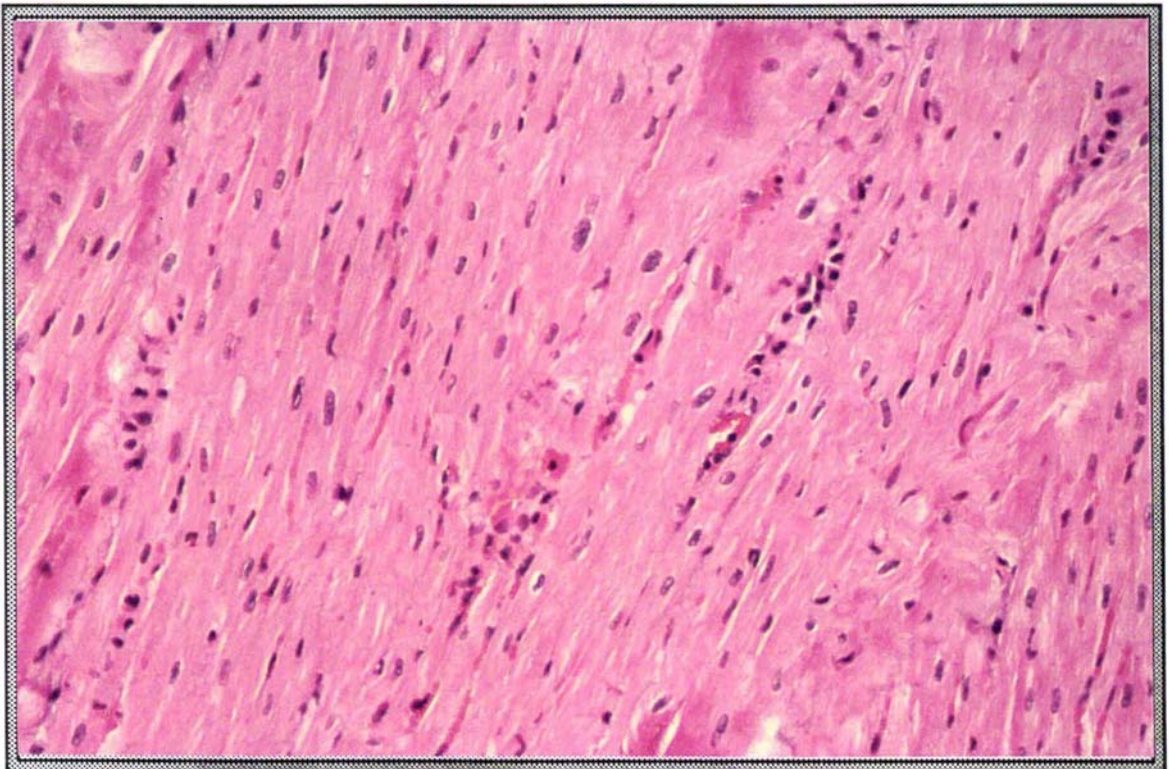
Focus of mononuclear inflammatory cells closely associated with the blood vessel endothelium and appearing to cause distortion and narrowing of the lumen.

Figure 4.9 Section of bladder from possum 3437; H & E stain (x 86).



Perivascular cuffs of mononuclear inflammatory cells in the sub-mucosa. Lymphocytes and plasma cells are the predominant cell types identified in the higher power insert (x 430).

Figure 4.10 Section of myocardium from possum 3437; H & E stain (x 215).



A few, small and scattered foci of mild non-suppurative myocarditis were identified. Similar foci were identified adjacent to the endocardium.

4.3.7 Other neurological lesions.

An incidental finding, in fewer than 5% of possums challenged with WPD, was the identification of protozoan tissue cysts in the neural parenchyma. These were not associated with an inflammatory response and were very rare in the possums in which they were found. On one occasion a wild, locally caught possum, with neurological signs indistinguishable from WPD, was submitted for investigation. The histological lesions consisted of a florid nonsuppurative meningo-encephalitis with prominent cuffs and several gliotic foci. Protozoan cysts were identified in all of the serial brain sections examined and also in the myocardium. Thorough observation of serial sections demonstrated a few tissue cysts at the centre of mild to moderate inflammatory reactions. The meningo-encephalitis was more severe than that seen in possums affected with WPD and the abundance of protozoal tissue cysts suggested a causative association. There were a few subcapsular foci of interstitial nephritis, swelling of the renal tubular epithelium and mild perivascular, lymphoid hepatitis. Changes in these organs were very mild and not considered typical of WPD.

4.3.8 Rabbits: Observations, clinical signs and pathology.

During the period of communal housing, two of the does became pregnant and one of these was sacrificed to provide tissues for inoculation into possums. The second pregnant female gave birth to five kits, none of which survived. Several of these were partially eaten and were removed from the enclosure. One of the smaller female rabbits was frequently bullied as evidenced by scratches and missing fur along the entire length of its back. This animal was eventually found dead with extensive loss of fur along the back and flanks. Scratches frequently penetrated the epidermis and significant exudation of tissues fluids and blood matted the remaining pelage. The limbs had been partially cannibalised. The other rabbits remained healthy.

No gross abnormalities were detected in rabbits that were electively killed and necropsied. Post-mortem examination of the rabbit that died revealed minimal abdominal fat reserves and bruising in the subdermis. Death was attributed to probable electrolyte disturbances and hypothermia from exudative, traumatic dermatitis. Lesions were estimated to cover > 25% of the body surface and bullying, probably as a result of social

stress, was implicated. No lesions or protozoal tissue cysts were detected in rabbit tissues that were examined histologically.

4.3.9 Inoculation of possums with rabbit tissues.

None of the possums inoculated with rabbit tissues developed clinical signs of WPD.

There were no gross changes at necropsy, no histological changes associated with WPD and no protozoal parasites detected.

Table 4.9 Numerical classification of WPD : Possums challenged initially and used for the filtration trial.

Possum Identity	Exposure Duration	Inoculum + Route	CLINICAL SIGNS				HISTOLOGICAL SIGNS					WPD Score
			Ataxia	Immobile	Behaviour	Appetite ↓	CNS	Liver	Kidney	Heart	Other	
LT*	14	3ml I116 tissues (ip)	+++	-	+	+	++	+	+	-	-	9
ST*	14		++	+	+	+	++	+	+	-	-	9
A*	25	10ml K078 and K096 tissues (ip)	++	+	+	+	++	+	+	-	-	9
B*	25		+++	-	+	+	++	+	+	-	+	10
C*	25		+++	-	+	+	++	+	+	+	+	11
Silver*	14 D	5ml LT and ST tissues (ip)	++	+	+	+	++	+	+	-	-	9
Black*	19		+++	-	+	+	++	+	+	-	-	9
667	29	filtrate	+++	-	+	-	++	+	+	+	+	10
668	27	filtrate	+++	-	+	-	++	+	+	-	+	9
669	16 D	supernatant	+++	-	+	+	++	+	+	-	+	10
3405	29	supernatant	+++	-	+	-	++	+	+	+	+	10
3437	15	homogenate	+++	-	+	+	++	+	+	+	+	11
3403	29	homogenate	++	+	+	-	++	+	+	+	-	9
636	46	-ve control	-	-	-	-	-	-	-	-	-	0
640	46	-ve control	-	-	-	-	-	-	-	-	-	0
666	46	-ve control	-	-	-	-	-	-	-	-	-	0

Wobbly possum disease (WPD) was established at Massey University when Chatham Island possums*, inoculated by the intra-peritoneal (ip) route with tissues from Invermay possums and presumed to contain an infectious agent, developed a severe neurological disease. Stewart Island possums (numbered) were then inoculated with an infectious spleen homogenate, supernatant or filtrate (0.22 µm) to ascertain whether or not the size of the infectious agent was consistent with a virus. In the filtration trial a separately caged, non-inoculated control possum was housed close to each treatment pair. Exposure duration = Number of days from inoculation or initial exposure to possums challenged with the infectious WPD agent until euthanasia or death (D). Appetite ↓ = Appetite decrease. + / ++ / +++ = Presence of a clinical sign or histological lesion associated with WPD. Changes indicating CNS involvement are weighted (4.2.6).

4.4 DISCUSSION

Wobbly possum disease is a recently recognised neurological disease of possums characterised by ataxia. Experimentally infected possums almost invariably develop severe behavioural and locomotory changes resulting in anorexia, ataxia, cachexia and death. Histological changes in the CNS are highly suggestive of a viral aetiology. This observation was supported in a carefully designed transmission experiment which reproduced WPD following filtration of a clarified tissue suspension from affected possums through a 0.22 μm membrane.

Bacterial and protozoal aetiologies were ruled out by the transmission experiments with filtered spleen. Mycoplasma infection was more difficult to definitively exclude by this method because mycoplasma are small (diameter of 0.3 - 0.8 μm), pliable and can pass through a 0.22 - 0.45 μm pore diameter filter (Yamamoto, 1990; Holt *et al.*, 1994). However, WPD was unlike any of the clinical disease entities associated with mycoplasma which are often considered weakly pathogenic in their own right and commonly cause serious disease only in combination with other aetiological agents (Blood and Henderson, 1983). When severe infections have been attributed to mycoplasma species, for example contagious bovine pleuropneumonia, contagious caprine pleuropneumonia and contagious agalactia of sheep and goats, certain mucosal and epithelial surfaces were characteristically involved and these included the respiratory and urogenital tracts, the mammary gland, synovial and conjunctival surfaces (Tully and Whitcomb 1979; Blood and Henderson, 1983). Mycoplasma associated meningitis has been recorded but not specific mycoplasma associated vasculitis or hepatitis (Brunner and Laber, 1985; Razin and Barile, 1985; Tully and Whitcomb, 1979). Mycoplasma infections, frequently implicated in human lower urinary and reproductive tract infections, occasionally progress to pyelonephritis and glomerulonephritis (Taylor-Robinson, 1985; Taylor-Robinson and McCormack, 1979). In contrast, interstitial nephritis and frequent vascular association was a constant feature of WPD. Prion diseases were ruled out, for although prions could easily pass through a pore size of 0.22 μm , there are no precedents for prions causing other than spongiform encephalopathies, as seen in scrapie-like diseases (Fraser, 1993), and none which have a short incubation period.

Thus, the WPD agent was shown to be of a size consistent with a virus. It was considered highly likely that WPD was a virus, rather than one of the infectious agents mentioned above and that the likelihood of a completely new type of infectious agent was

remote. Research elsewhere has since provided further evidence for a viral aetiology (O'Keefe, 1996; O'Keefe *et al.*, 1997).

Some haematological and biochemical abnormalities have been reported to be associated with WPD (O'Keefe *et al.*, 1997). These were an increase in total protein, a decrease in serum albumin and an increase in serum globulins, an increased number of circulating nucleated RBCs and a decrease in the number of eosinophils. Creatinine levels were decreased. Most of these trends were also detected in the series of possums from the current study.

The most apparent haematological changes detected in the current study and not previously described were the increased frequency of aberrant erythrocytes with abnormal morphology and the appearance of large, atypical (activated or reactive) lymphocytes. Affected possums had a significantly increased number of large, atypical lymphocytes and (although of no statistical significance) a concurrent decrease in the number of normal lymphocytes. There was no difference between groups in the size of the total lymphocyte pool but there appeared to be functional changes within the lymphocyte pool of affected possums. It was difficult to morphologically differentiate atypically large lymphocytes from monocytes using diffquik staining. Therefore these leukocyte categories were pooled for affected and normal possums to confirm a highly significant difference in the number of large lymphoid or monocytic mononuclear cells (Table 4.8). These cells were hypothesised as having become specifically up-regulated in response to antigenic (agent) stimulation and phagocytic requirements respectively. Atypical lymphocytes (and/or monocytes) were detected histologically in the meningeal microcirculation of possums with WPD suggesting that they were unlikely to be an artefact of blood smear preparation.

The current study records a statistically significant anaemia following clinical and histologically confirmed WPD infection. Haematocrit, RBC and haemoglobin concentration of affected possums were below published reference values (Fitzgerald *et al.*, 1981; Presidente and Correa, 1981; Presidente, 1982; O'Keefe and Wickstrom, 1998). O'Keefe *et al.*, (1997) recorded a decrease in the mean haematocrit and haemoglobin concentration of possums affected with WPD. Although their findings were not statistically significant, the current study suggests these results probably reflect a genuine pathological process. Other quantifiable indicators of changes to the erythron

(MCV and MCHC) were not reported by O'Keefe *et al.* (1997) and were difficult to interpret in the current study. Reticulocytes were not enumerated as an indication of bone marrow response but polychromasia (subjectively assessed) and nucleated RBCs (statistically significant) were evident features of WPD that may be interpreted as a response to an anaemia even though the mechanisms have not been specifically identified in WPD. The anaemia associated with WPD infection was considered to be due to the presence of the infectious agent but the chronicity of the disease process (many possums were infected for one month or more) may have exacerbated these findings.

Hyperproteinaemia due to hyperglobulinaemia has been previously reported (O'Keefe *et al.*, 1997) and is consistent with the acute phase of an inflammatory process and frequently associated immune response in domestic animals (Tizard, 1992; Cotran *et al.*, 1995). In WPD, the relative predominance of γ globulins, the presence of large, reactive lymphocytes in blood films and plasma cells in histological sections supported activated immunological (humoral) processes. Total serum protein, β and γ globulin concentrations were increased compared with published reference ranges (Fitzgerald *et al.*, 1981). Severely affected possums were in very poor condition when humanely killed and dehydration may have artificially elevated total protein and globulin values in some individuals. Total protein concentrations in the current study were higher than have been reported by O'Keefe *et al.* (1997). These authors also reported a significant decrease in albumin concentrations which, in contrast, was within normal limits in the current study. Dehydration could account for these differences and if dehydration was a significant feature of affected possums at the time of euthanasia and blood collection, then a more significant anaemia and possibly hypoalbuminaemia may have been masked.

In the statistical analyses on the above data, the non-parametric test was included because it is robust to outlying values and assumptions about non 'normal' data distribution. Non-parametric tests are not as powerful as parametric tests and neither statistical test is able make definitive statements when sample sizes are small (Motulsky, 1995). The analyses performed here provide an initial summary of trends that could be further investigated with a larger group of clinically affected possums. Reference possum blood for the current study came from possums prior to inoculation whereas in the O'Keefe series, wild caught possums and sentinel possums were used as well as possums prior to inoculation. Marked age changes and sexual dimorphism for haematological values in possums have been previously documented (Fitzgerald *et al.*, 1981; Presidente and Correa, 1981) and

greater standardisation has the potential to improve the detection and interpretation of changes due to infection with WPD. In particular, control possums should be subject to identical husbandry conditions and all possums acclimatised to the housing prior to the start of experimentation (Buddle *et al.*, 1992). Evaluation of possum bone marrow responses following infection with WPD, including descriptive baseline studies for wild captured and captive possums, would be a worthwhile future study.

Notwithstanding the statistical disclaimer for the small numbers of possums studied, it is reasonable to speculate that the anaemia of WPD may be mediated through immune and/or mechanical phenomena. High levels of circulating γ globulins are presumably synthesized in response to antigenic stimulation. Any association of specific antigens with possum RBC (the presence of antigen/infectivity in blood has been demonstrated, chapter 6) can lead to antibody mediated lysis via the complement pathway (Tizard, 1992). For example, equine infectious anaemia virus provokes a strong humoral response in immunocompetent horses and the anaemia is due in part to complement (C3) mediated intravascular and extravascular haemolysis (Cook *et al.*, 1996). Similar mechanisms have been described for RBC destruction in auto-immune haemolytic anaemia. Schistocytes and spherocytes have been associated with these processes but the latter may not be readily demonstrated in many animal species (Jain, 1986).

In many tissues the presence of intravascular and perivascular accumulations of inflammatory cells, closely associated with and beneath the endothelial surfaces of vessels, suggested that vasculitis may have been a primary lesion in WPD. Therefore, another possible reason for the appearance of poikilocytes and schistocytes was mechanical damage to erythrocytes that moved through affected capillaries. These mechanisms have been proposed for processes such as DIC and microangiopathic hemolytic anaemia (haemolytic uraemia syndrome, thrombotic thrombocytopenia and severe burns) (Jain, 1986; Payne *et al.*, 1995; Rozenberg, 1996). Direct evidence and/or specific tests to implicate a contribution from these mechanisms in the anaemia of WPD await further critical experimentation.

Wobbly possum disease was associated with characteristic histological lesions in the CNS, liver, kidney and many other tissues. Inflammation was invariably mononuclear with a prominent vascular association in most tissues. At a single tissue level no lesions were pathognomonic. Altogether, however, the widespread and characteristic pattern of

inflammation in tissues from possums experimentally infected with WPD was unlike any previously described disease of possums and histological diagnosis of WPD was both feasible and currently realistic.

The haematology, serum protein electrophoresis and histopathology of WPD was not unlike other known viral diseases. Systemic plasmacytosis and gammaglobulinaemia were shown to be the underlying cellular and molecular events leading to immune complex glomerulonephritis and arteritis in parvovirus induced, Aleutian disease (AD) of mink (Henson *et al.*, 1969; Porter *et al.*, 1969). A form of an immune mediated coronavirus disease of cats, feline infectious peritonitis (FIP), has been reported in which neurological signs predominate. Plasma cell infiltrates in the CNS and other tissues accounted for the gammaglobulinaemia but other cell types contributed to a pyogranulomatous meningitis (Summers *et al.*, 1995). The cellular make up of WPD lesions involved fewer plasma cells and no neutrophils compared to the lesions observed in AD and FIP respectively. Lymphocyte and plasma cell reactivity plus a broad hyperglobulinaemia suggested B-cell proliferation and immune phenomena contributed substantially to the pathogenesis of WPD lesions. However, there was little evidence for immune complex deposition or necrosis and destruction of target cells

The cell types and venous bias of inflammatory reaction in the CNS was similar to Borna disease (BD) originally described more than 90 years ago by Joest and Degen "*The inflammatory cells appear in the form of perivascular cuffs of various widths around venules and small veins, and, rather infrequently, around small arteries. They occupy, as a rule, the adventitial space of the vessels but occasionally break through the adventitial membrane to form perivascular infiltrates. The bulk of the infiltrating cells consist of lymphocytes, monocytes, and to a lesser extent plasma cells. Polymorphonuclear leukocytes have rarely been seen*" (Gosztonyi and Ludwig, 1995). A significant range of mammalian host species have been infected by BD and it is reported that the virus or close relatives of the agent can infect birds as well (Rott and Becht, 1995; Malkinson *et al.*, 1995). Within the many host species, an equal range of clinical signs, histopathological lesions and disease outcomes, from unapparent infection to debilitating neurological disease and death, have been reported (Rott and Becht, 1995). Many features of WPD were atypical of BD, in particular the wide extra-neural tissue tropism, but the venous bias of the inflammatory lesions in the CNS was similar.

Perivascular, lymphoid accumulations in addition to necrosis of vessel adventitia have been described in herpesvirus-associated, malignant catarrhal fever (MCF) of cattle (Summers *et al.*, 1995). The primary vascular association was emphasized and relatively mild degenerative changes and reactive gliosis in neural tissue did not indicate tropism for the CNS. Lesions associated with blood vessels were seen in many tissues in possums infected with WPD. Neural lesions were relatively mild, gliosis extremely rare and necrosis was not detected in association with lesions in any tissue. Therefore the CNS may not be a target tissue for WPD *per se* but just one of many tissues in which lesions associated with blood vessels are manifest.

Atypical lymphocytes were a prominent feature of WPD. While possum lymphocytes have been shown to be variable (Barbour, 1972), increased size and irregular nuclear morphology (including binucleate forms) was associated with WPD infection. It has been demonstrated that a subpopulation of CD8⁺ cytotoxic T-cells (lymphoblasts) characterise the atypical lymphocytes of infectious mononucleosis (Epstein Barr virus, human herpesvirus-4) following stimulation of B-cell class-1 MHC receptors by viral antigens (White and Fenner, 1994). A similar mechanism for the pathogenesis and immunopathology of MCF has been demonstrated (Nakajima *et al.*, 1992). Evolutionary conserved, polypeptide sequences of human T-cell and B-cell associated molecules have been used to raise cross reactive antibodies and these in turn have been used to immunohistologically investigate possum and other marsupial lymphocytes (Canfield and Hemsley, 1996; Hemsley, *et al.*, 1995). Not only have atypical or activated lymphocytes been shown to be a feature of certain viral diseases, but techniques to investigate the lymphocyte subsets involved, including those of possums, have been demonstrated.

Another feature of WPD was the lack of significant necrosis in association with observed lesions. Without infiltrating lymphoid cells there would be few detectable changes in tissues. The profound depression of many possums with advanced WPD infection was not accounted for by overwhelming histological changes in affected tissues and loss of organ reserve appeared unlikely. The host inflammatory response to cytomegalovirus (CMV or human herpesvirus-5) infection, which consisted of plasma cell and lymphocyte infiltration (vasculitis) plus viral cytopathology, presented a similar paradox as stated (Arlford and Britt, 1990). *"Even with generalised lethal disease, the number and distribution of typical lesions in affected organs does not suggest the level of functional disturbance."* It could be suggested that the primary lesion of WPD is also a form of

vasculitis in which infiltration by mononuclear cells predominates over virus or host induced cell destruction. The pathogenesis of CMV and complex mechanisms of latency are incompletely understood (White and Fenner, 1994) and unknown factors, other than specific viral cytolysis, are speculated to bring about organ dysfunction. However, clinically severe manifestations of CMV in the foetus and immuno-compromised host (AIDS patients and graft recipients) suggests that the immune response mediates eventual clearance, not immune complex disease.

Many possums experimentally infected with WPD became progressively ataxic, emaciated and died and it appeared that a further significant number would have died of the disease process if not humanely killed. It was not determined which lesions associated with WPD caused death and whether or not functional consequences were sufficient to cause high mortality in the wild. Whilst brain lesions were not extensive in terms of inflammatory cell volume, these changes may have been important in determining outcome by compromising host behaviours and survival fitness (adaptation to captivity).

While a functional and up-regulated humoral immune response was characterised by plasmacytosis and gammaglobulinaemia, it appeared that antibodies were unable to clear experimental infection which frequently ran for four weeks. Lymphocyte sub-populations and the role of cell-mediated responses in the pathology and/or eventual clearance of WPD are unknown. What is the inherent pathogenicity of the virus with respect to the severity and duration of clinical signs and lesions? What are the mechanisms by which the virus interacts with the host immune system? and why does a seemingly strong immune response fail to prevent death in a significant number of possums? It is clear that the immune response of the possum to WPD is of fundamental importance. Immuno-modulating drugs could be rationally employed to investigate the contribution of host responses to WPD histopathology. For example, cyclophosphamide has been shown to indefinitely prevent the effects of virus-induced Borna disease encephalitis in four week old rats (Narayan *et al.*, 1983). Irradiation protected mice from lethal lymphocytic choriomeningitis (LCM) virus infection and lethal LCM was induced in cyclophosphamide treated carriers by adoptive transfer of LCM virus-immune spleen cells (Lehmann-Grube, 1982). Lesions of AD of mink were prevented by immunosuppression with cyclophosphamide and enhanced by immunisation with inactivated virus vaccine followed by live virus challenge (Pearson and Gorham, 1987).

The pathogenesis of WPD virus could only be speculatively proposed on the available evidence. A period of replication in possum white blood cells (WBC) was possible, based on a demonstrated viraemia. The abnormal morphology of lymphocytes from affected possums and the fact that viruses have been shown to replicate and/or be distributed in WBCs (Tyler and Fields, 1990) also supports this hypothesis. Alternatively, viral products expressed in target tissue(s) could strongly interact with and influence the immune cell response (notably lymphocytes). To explain the wide distribution of lesions in WPD it was suggested that either numerous tissues supported virus replication or that a single, widely distributed cell type, for example vascular endothelium, was the preferred host cell.

Further studies are required to determine the pathogenesis of WPD and the above discussion has indicated a number of potentially similar viral diseases, including immune complex diseases, that could be used as research models. While tools to characterise the atypical lymphocytes seen in WPD are available (Hemsley *et al.*, 1995), specific reagents (immunological and/or nucleic acid probes) and a considered experimental design are required to fully investigate temporal and spatial events following WPD infection.

Although WPD causes characteristic lesions in the kidney (4.3.6), interstitial nephritis in possums has been previously associated with leptospirosis (Durfee and Presidente, 1979a; Hathaway, 1981), and is a frequently described incidental finding (Cooke, pers. comm.) potentially requiring differentiation from lesions induced by WPD. Mononuclear inflammatory cells associated with cortico-medullary blood vessels were similar to the vascular lesions seen in other tissues but were often less prominent than generalised interstitial nephritis. Control possums did not have vascular lesions and the occasional focus of mild, sub-capsular interstitial nephritis was clearly different from the generalised interstitial nephritis of inoculated possums. Mild renal lesions in young possums were unlikely to be due to leptospirosis, as infection is associated with sexual maturity and seroconversion at approximately 18 months of age (Durfee and Presidente, 1979). While moderately severe interstitial nephritis in adult possums, in association with clinical signs and other histological lesions of WPD, was more likely to be due to inoculation with WPD than an incidental finding, possums for some of the transmission studies (chapter 6) were captured from areas where leptospirosis was prevalent (Horner *et al.*, 1996). Where this likelihood existed it was possible that the kidney lesions associated with WPD were superimposed on pre-existing lesions of interstitial nephritis due to leptospirosis.

This was improbable for possums used for the initial trials as there is no serological evidence that leptospirosis is present in possums from off shore Islands (Horner *et al.*, 1996). The absence of leptospire in histological sections from these possums was not unexpected and WPD was demonstrated to cause a significant nephritis in its own right. Where possums for transmission trials were obtained from areas with a high sero-positive prevalence of leptospirosis it was considered possible, but unlikely, that WPD induces a significant recrudescence of leptospire associated nephritis. More rigorous protocols would be required to examine this possibility.

Wobbly possum disease also induced characteristic lesions in the liver (4.3.6). Extensive perivascular, mononuclear cuffs invading the luminal surface and occupying intra-parenchymal sites differentiated WPD from mild lymphoid hyperplasia, seen in a proportion of wild caught possums.

In contrast with the liver and kidney, no pre-existing CNS inflammation was recorded in wild caught possums used as controls. One clinically ataxic, wild caught possum was differentiated from experimental WPD on histological differences observed in the pattern of inflammation in the CNS, the detection of protozoal tissue cysts in association with encephalitic lesions and a lack of significant changes in the liver or kidney (4.3.7).

The scoring system successfully overcame minor difficulties of histological interpretation when one or two tissues from young possums were mildly affected (neurological indicators present). For example, joeys with WPD were diagnosed by scoring the CNS lesions as positive when mild, discrete foci of typical inflammatory cells in the meninges (often without accompanying encephalitis) indicated a significant CNS lesion. Scoring minimal CNS lesions as negative (paucity of typical inflammatory cells) when clinical signs were present, implied that the numerical diagnosis of WPD relied on clinical indicators and changes in tissues other than the CNS (4.2.6). When CNS lesions were mild or equivocal, histopathological changes in the liver and kidney were as characteristic of the pathology of WPD as the neurological lesions themselves and were important for diagnosis. Subsequently, a young, individually caged, control joey thrived on the standard diet. This indicated that WPD, and not diet or husbandry factors, was responsible for the clinical signs and often insubstantial histopathology in this age group of possums.

The use of a scoring system to collate data with a subjective element was not expected to perform with 100% precision. Any numerical system to define a disease process must be recognised as a construct and likely to be tested with cases that are difficult to differentiate. The scoring system, as the "test" of other collated data, could be assessed in terms of sensitivity and specificity once reliable assays for the causative agent or a WPD-specific immune response are available. Borderline cases of WPD (false positives and false negatives) could be specifically tested, sero-epidemiological studies undertaken and archival material investigated with the appropriate tests. Until such time, substantiation of WPD is based on clinical and histological correlates as defined above.

Case definitions have been widely used for describing newly recognised disease syndromes and a relatively recent example of this precedent occurred with the acquired immunodeficiency syndrome (AIDS). The first 1000 cases of AIDS were summarised and collated by the Centers for Disease Control (CDC) and a relatively minimal case definition adopted. AIDS was defined as '*the occurrence of biopsy proven Kaposi's sarcoma or biopsy/culture proven infection at least moderately predictive of cellular immune deficiency in patients without pre-existing immunosuppressive disease*' (Jaffe *et al.*, 1983). Case definitions were reviewed, expanded and became more elaborate after discovery of the human immunodeficiency virus (HIV) and HIV testing became available (anon, 1987). Once the aetiological agent of AIDS had been identified and suitable tests developed, the need for reliance on such diagnostic constructs was past.

4.5 SUMMARY

A viral aetiology for WPD was strongly supported when filtered infectious material (clarified spleen suspension from a confirmed case of WPD passed through a 0.22 μm membrane) could transmit disease to susceptible possums following intra-peritoneal inoculation. Infected possums developed behavioural changes culminating in profound ataxia two to four weeks after inoculation with infectious tissues. Interactive responses and spontaneous movements were reduced. Many possums became anorectic, weak and eventually cachectic. Anaemia and hyperglobulinaemia were recorded in clinically affected possums. Apart from loss of body condition and fat reserves there were no gross findings at necropsy.

The histologically significant lesion supporting ataxia was a nonsuppurative meningo-encephalitis. Plasma cells and lymphocytes were the predominant inflammatory cells seen both diffusely and with a vascular association in the meninges but encephalitis was almost exclusively associated with blood vessels. Characteristic lesions displaying a similar cellular make-up and often with a vascular association were detected in the liver, kidney, heart and bladder. Mononuclear inflammatory cells were seen in interstitial locations in the kidney, bladder and heart and an increase in the number of nucleated RBCs in addition to parenchymal foci of mononuclear cells was detected in the liver. Wobbly possum disease is characterised by multiple organ disease and may be more accurately described as a pan-vasculitis than an encephalitis, in spite of its clinical presentation and 'descriptive' name.

Histological lesions and hyperglobulinaemia suggest that the pathogenesis may involve immune mechanisms in the absence of obvious cytolysis or necrosis. Although histological lesions do not always appear extensive, in the laboratory situation WPD is a severe, progressively fatal disease. For this reason WPD virus is worth further investigation as a potential vector or disease agent for the biological control of possums in New Zealand. In addition to characterisation of the agent and determination of its prevalence, further investigation of the pathogenesis of WPD would be greatly enhanced by the development of specific reagents to follow the disease course in possums.

Chapter 5

WOBBLY POSSUM DISEASE : THE AGENT

5.1 INTRODUCTION

The histological appearance of lesions associated with wobbly possum disease (WPD) reported in this study and by others (Macintosh *et al.*, 1995) suggested a viral aetiology. This view has been subsequently supported by a filtration study (chapter 4). The ultimate characterisation and classification of the WPD agent would be hastened, as would many other aspects of research, if an isolate of WPD could be adapted and propagated *in vitro*. Thus attempts to propagate the virus *in vitro* and studies to determine the morphology of the agent were undertaken.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of WPD infected tissues and homogenates for EM.

(i) Routine EM procedure : Suspensions of liver, spleen and brain tissues from WPD infected possums K0078 and K0096 (4.2.1) were clarified by centrifugation at 2,700 x g for 30 minutes. Supernatants were collected and 4 ml overlaid on a 1 ml cushion of 45% (w/w) sucrose in 6 ml Beckman ultracentrifuge tubes. These preparations were ultracentrifuged at 180,000 x g for 2 hours, the supernatants discarded and the pellets resuspended in distilled water (dH₂O) overnight. Negative staining was performed as described in section 3.2.10.

(ii) Density gradient centrifugation : Continuous gradients were prepared from both sucrose and caesium chloride (CsCl) to cover a wide range of virus particle densities. For CsCl, solutions of 1.2 and 1.4 g/cm³ were mixed in equal 15 ml proportions and layered using a gradient maker into a 40 ml Beckman ultracentrifuge tube. The same technique was used to prepare sucrose gradients from a 66% (w/w) sucrose stock made up in GTNE buffer (Glycine / Tris / NaCl / Edta) (appendix 3). Sucrose gradients ranged from 20% - 55%. Five mls of standard inoculum (6.2.1) was layered on top of

each gradient and centrifuged at $80,000 \times g$ for 16 hours. Two bands were observed in both the sucrose and CsCl gradients and these were collected by side puncture of the centrifuge tube using a 23 g x $1\frac{1}{2}$ " needle. Replicate gradients were carefully fractionated into 1.5 ml aliquots by puncture of the base of the centrifuge tube with a 25 g x $\frac{5}{8}$ " needle. Gradient fraction density (g/cm^3) was confirmed by weighing aliquots from each fraction.

(iii) Preparation of gradient material for electron microscopy : Bands collected from both sucrose and CsCl gradients were diluted in GTNE to a volume of 4 ml and particulate material pelleted by ultra-centrifugation ($100,000 \times g$: two hours). The supernatant was discarded and the pellet resuspended overnight in dH_2O . Fractions recovered from fractionated gradients were labelled and stored at -70°C . Caesium chloride was subsequently removed by placing an aliquot (75 - 100 μl) onto a millipore nitrocellulose membrane (0.025 μm : Millipore corporation) floating on 500 ml of dH_2O for two hours. Sucrose was subsequently removed from 1.5 ml fractions by placing the entire sample into dialysis tubing (MW cut off : 6 - 8,000 daltons). Three 500 ml changes of dH_2O at 8 - 12 hour intervals completed the dialysis. Concentration of samples following volume increases was achieved by ultra-centrifugation as described above. All samples were negatively stained as previously described (3.2.10).

(iv) Immune-Electron Microscopy (IEM) : A second preparative technique sought to immunologically concentrate the virus for electron microscopic identification using protocols based on the 'serum in agar' method (Hayat, 1989). Possum 618 (infected by natural contact) was 'vaccinated' with a 1 ml dose of formalin inactivated standard inoculum (chapter 6) by intra-muscular injection and convalescent / hyperimmune serum collected one month later. Convalescent serum from possum 615 (also infected by natural contact) or possum 618 was added to 1.25% agar (Difco, Michigan) once it had cooled to 45°C . A 1:10 ratio of serum to agar was used for samples that were taken from obvious bands and a 1:50 ratio used for samples from the fractionated gradients. Individual 200 μl cups in a flexible microtitre plate (Falcon Microtest III, Becton Dickinson) were three quarters filled with the 'serum in agar' mixture and stored overnight (4°C). A 30 μl sample was then placed directly onto the agar surface and

formvar coated, BSA treated copper grids were placed coating side down onto the sample and incubated for 1 hour at 37 °C. Grids, adhered to the agar surface during incubation, were refloated by the addition of a drop of dH₂O, washed three times by floating on drops of dH₂O and negatively stained.

(v) Positive control for Immune-Electron Microscopy : To assess the serum in agar preparative technique, convalescent equine serum with a neutralising titre of 1/128 to equine herpesvirus-1 (EHV-1) was incubated with filtered supernatant from EHV-1 infected equine foetal kidney cells. The virus preparation (2×10^6 TCID₅₀ / ml) was diluted 1:3 in dH₂O prior to filtration through a 0.22 µm filter. Allowing for some loss of titre with filtration, the estimated concentration of EHV-1 was 5×10^5 TCID₅₀ / ml. Serum was diluted in agar to give 1:10, 1:20, 1:40 and 1:60 ratios and samples prepared as described above. Visibility of EHV-1 or virus particle clumping in a filtered sample of EHV-1, routinely prepared, was compared with EHV-1 visibility and clumping in the samples prepared by the serum-in-agar method. Assessment of the difference in visibility following use of the serum-in-agar method was subjective. Antisera to EHV-1 and EHV-1 antigen was kindly supplied by J. J. Donald and M. Dunowska respectively.

5.2.2 Inoculation in tissue culture.

(i) Standard protocol : Tissues from selected clinical cases of WPD were inoculated onto and co-cultivated with cell monolayers using methods previously described (chapter 3). Briefly, tissues (liver, kidney, spleen, lung, brain) from Invermay possum I116 were prepared as 10% suspensions in MEM using a Tenbroek tissue grinder and further diluted to 1:100. Aliquots (100 µl) of each tissue at both dilutions were inoculated onto PTK2, PRT-1/2 and OPK cells in 24 well multiplates. Finely minced suspensions of liver, kidney, spleen, lung and bone marrow were explanted onto the same cell lines.

In a second experiment, brain, liver and spleen samples from possums A, B, and C, killed 26 days post-inoculation and confirmed to have WPD, were prepared as 10% tissue suspensions in MEM. A reduced volume (~50 µl) of inoculum was co-cultivated with the monolayer cells by drop-wise addition of cells and tissue debris until the monolayers were 80% obscured. In addition to PTK2, PRT-1/2 and OPK cells, a lower passage

(p18), more fibroblastic strain of marsupial heart cells (CSL-223: Commonwealth Serum Laboratories, Australia), a lower passage (p5) of PRT-1/2 cells and a primary possum kidney cell culture (p1) were used. In keeping with established protocols, three seven day long passages, interrupted by two freeze thaw cycles, were undertaken. Haemadsorption and chamber slide inoculation experiments were performed on third pass material. Isolation was considered negative if no cytopathic effect (CPE) or haemadsorption could be detected by these methods. Primary cell cultures of kidney tissue from possums A and C (Dispase disaggregation 3.2.7) were established to detect spontaneous deterioration.

(ii) Infected possums killed at different times post-inoculation : One possum (3437) was killed as early as clinical signs of WPD were definitively established (14 days post-inoculation) and a second possum (668) killed ten days following the onset of clinical signs (27 days post-inoculation). Liver, spleen and brain tissues (10% suspensions, dropwise addition as above) from these two possums were co-cultivated with nine different cell lines (PRT-1/2, OPK, PTK2, MDBK, MDCK, NLFK, RK13, VERO and BHK) and three primary cell monolayers (PPK, PCK and PCEF) (table 5.1). Primary possum kidney cells (PPK) were prepared by the dispase method (3.2.7) and primary chicken kidney (PCK) cells and primary chicken embryo fibroblasts (PCEF) by trypsinisation (3.2.7). PPK cells were established in small flasks and passed once to form monolayers in 24 well plates. All other cells with the exception of hamster kidney (BHK) cells, which grew better in small flasks, were grown and inoculated in 24 well multiplates. The growth medium, maintenance medium and primary medium for cultivation of these monolayers has been previously described in section 3.2.2. Equine serum (5%) was used to supplement MDBK cells. Four one-week passages were performed and haemadsorption and chamber slide inoculations were performed on fourth passage material.

(iii) Primary chicken embryo fibroblasts and baby possum kidney cells : A further isolation attempt was made using PCEFs and 'embryonic' primary cells derived from the kidneys of pouch dependent possum joeys (2 - 3 months). Baby possum kidney (BPK) cells and PCEFs, prepared by dispase digestion and trypsinisation respectively (3.2.7), were used as confluent primary monolayers in small flasks.

Liver and spleen tissues were collected from a freshly killed possum (Wallaceville 6C) with clinical and histologically confirmed WPD. These tissues were chilled (4 °C), couriered to Massey University and processed immediately. Stored (-70 °C) liver from possums 668 and 3437 and stored (-70 °C) spleen and brain from possum 667, all confirmed to have WPD by histological examination (4.2.9, 4.3.2), were thawed at room temperature and processed immediately. Liver and spleen tissues from possum 6C were prepared by both mincing (to produce live cells) and complete tissue disruption in a Tenbroek grinder. Tissue homogenates were pooled and adjusted to approximate a 10% suspension in MEM containing 1% FBS and 1% PSK. Aliquots of up to 200 µl were added dropwise and adsorbed onto near confluent PCEF and BPK monolayers for one hour. Maintenance medium was replaced and flasks incubated as previously described. Liver tissue from possums 668 and 3437 and spleen and brain tissues from possum 667 were disrupted in a Tenbroek grinder to prepare 10% suspensions as described above. Separate flasks of near confluent PCEF and BPK were inoculated with up to 200 µl of infectious tissue suspension from each possum as described above. Cultures were maintained for one week with a single, partial media change after four days. Monolayers were checked twice weekly and frozen and thawed twice before being passed onto fresh monolayers. Seven passages were performed in PCEFs and four in BPKs. A duplicate set of PCEF monolayers were produced for the fourth passage and, following inoculation with material from passage three, these flasks were maintained with partial media changes until the monolayers deteriorated.

5.2.3 Inoculation in insect tissue culture.

A mosquito cell line (C6/36 *Aedes albopictus*) was kindly provided by Dr Terry Maguire, University of Otago. Mosquito cells were cultivated using RPMI medium (Gibco, BRL) supplemented with 5% FBS for growth, 1% FBS for maintenance (antibiotics and glutamine as previously described, appendix 1) and incubated at 28 °C with 5% CO₂ in a humidified atmosphere. Growth medium was removed, a 500 µl aliquot of the standard inoculum (6.2.1) adsorbed onto confluent C6/36 monolayers in small flasks (30 minutes) and maintenance media replaced. Six one-week passages, separated by two freeze thaw cycles, were performed and monolayers examined twice weekly for viral CPE.

5.2.4 Inoculation into mice.

A litter of day-old mouse pups was challenged with 0.01 and 0.03 ml of the standard inoculum (6.2.1) by the intra-cerebral and intra-peritoneal routes respectively. Mice were observed twice daily for the first week following inoculation and daily for a further three weeks. No further passages were performed.

5.2.5 Inoculation into eggs.

Two 11-day-old embryonated eggs were inoculated with 0.5 ml of the standard inoculum (6.2.1) via the chorio-allantoic membrane and two by yolk sac injection. Embryos and their membranes, harvested after a 10 day incubation, were visually inspected, examined histologically and passaged a second time by the same routes. Embryos and membranes from the second passage were assessed in the same manner.

Table 5.1 Celllines, cell strains and primary cells used in WPD isolation attempt.

Cell "line" abbreviation	Description species of origin	Cell morphology passage (p) details
PPK	Primary (1 ⁰) possum kidney	fibro-epithelial p = 0 to 3
BPK	Baby possum kidney (1 ⁰)	fibroblastic p = 0 to 2
PCK	Chick kidney (1 ⁰)	fibroblastic p = 0 to 2
PCEF	Chicken embryo fibroblast (1 ⁰)	fibroblastic p = 0
PRT	Possum reproductive tract	epithelial p = 5 to 25
OPK	Opossum kidney	epithelial p > 50
PTK2	Potoroo kidney	epithelial p > 75
CSL 223	Marsupial heart	fibroblast p > 18
MDBK	Bovine kidney	epithelial p > 40
MDCK	Canine kidney	epithelial p > 40
NLFK	Feline kidney	fibroblast p > 80
RK13	Rabbit kidney	epithelial p > 50
VERO	African green monkey kidney	epithelial p > 50
BHK	Hamster kidney	epithelial p > 50

5.3 RESULTS

5.3.1 Electron microscopy.

(i) Virus-like particles in preparations of tissues from WPD infected possums :

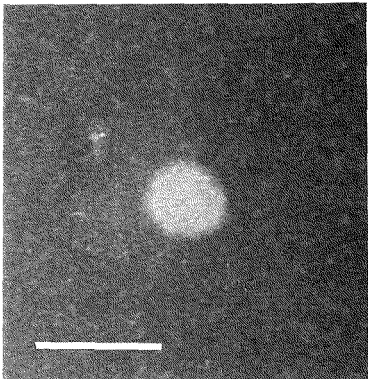
Using a serum to agar ratio of 1:50, virus-like particles were identified in CsCl fraction seven (1.28 g/cm^3) that were circular and had a diameter of 60 nm (figure 5.1). A second particle with a diameter of 60 nm was identified in the lower opalescent band of a CsCl gradient, which corresponded to fraction seven from the measured gradient (1.28 g/cm^3), and was prepared for EM using a serum to agar ratio of 1:10 (figure 5.2). This particle had the suggestion of hexagonal symmetry, a slightly irregular, electron lucent outline (fringe) and some evidence of internal capsid-like substructure. Faint, irregular surface projections could be identified.

Using standard negative staining, virus-like particles were identified in CsCl fraction 11 (1.22 g/cm^3), that had a roughly spherical outline and a diameter of 100 - 120 nm (figure 5.3). Stain penetration of one such particle (figure 5.3) revealed an irregular capsid-like internal surface and some evidence for a closely associated envelope with regular, short projections (8-15 nm) from the outer surface. Also in this sample (CsCl fraction 11, 1.22 g/cm^3), was a spherical particle in the same size range with less internal detail visible (figure 5.4). No VLPs were observed in material collected from sucrose gradients or following standard E.M. procedures on non-gradient derived samples.

(ii) Positive control - Immune-Electron Microscopy : Typical herpesvirus particles were identified in the positive control. At a serum to agar ratio of 1:60, enveloped EHV-1 particles were identified with a very broad, electron lucent halo that separated the viral envelope from the background stain (figure 5.5). Capsids were seen in adjacent pairs and as clumps of capsids together with disrupted capsid debris (figure 5.6, 5.7). At a serum to agar ratio of 1:10, an EHV-1 capsid was identified by an irregular, electron lucent tegument which may have been prominent due to the presence of antibodies (figure 5.8). Individual herpesvirus particles, but neither aggregations of particles nor electron lucent halos, were seen in the tissue culture preparation of EHV-1 stained using standard protocols.

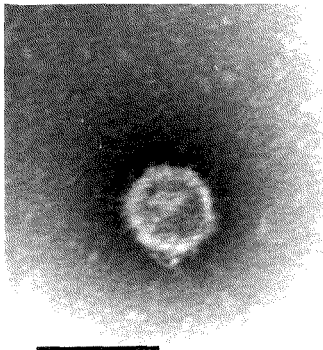
Figures 5.1 - 5.4 Negatively stained candidate virus particles for the WPD agent.
Magnification and bar representing 100 nm are supplied for each figure.

Figure 5.1



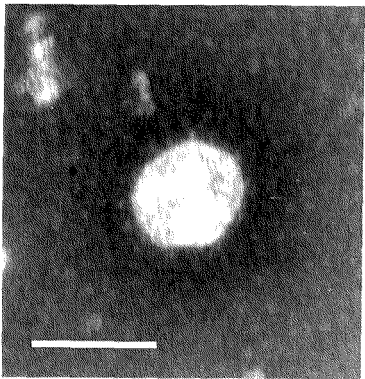
162,400 x

Figure 5.2



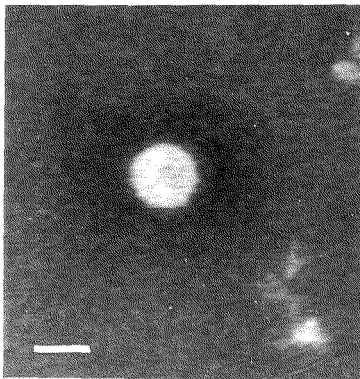
162,400 x

Figure 5.3



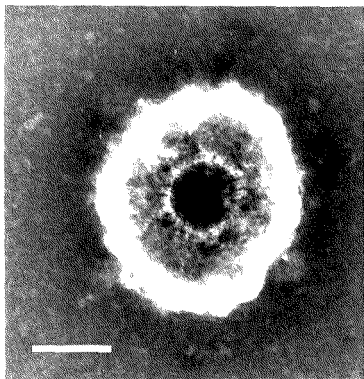
162,400 x

Figure 5.4



72,100 x

Figure 5.5



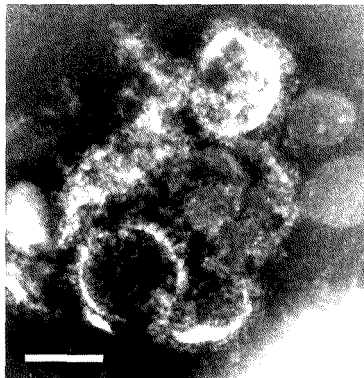
103,600 x

Figure 5.6



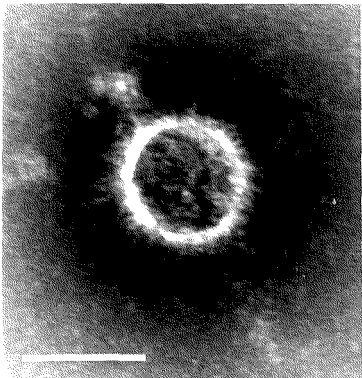
162,400 x

Figure 5.7



103,600 x

Figure 5.8



162,400 x

Figures 5.5 - 5.8 Negatively stained EHV-1 particles prepared using Immune-EM.
Magnification and bar representing 100 nm are supplied for each figure.

5.3.2 Attempted isolation of the WPD agent.

Mammalian Tissue Culture : No cytopathic agent was detected in any of the tissue culture systems used. Variable monolayer deterioration was detected in both the PCEFs and the primary possum cells. These changes took the form of "shrinkage" plaques (no visible cytopathology adjacent to plaques) and peripheral decreases in monolayer confluency in which cell elongation (partial retraction from culture surface) and monolayer detachment was noted. Almost invariably these latter changes occurred around the edges of wells. Further passage and examination by EM failed to reveal any repeatable cytopathic effect or virus-like particles in inoculated cultures. No haemadsorption and no abnormal cytopathology in chamber slides was detected.

5.3.3 Insect tissue culture.

No CPE was detected in any of the C6/36 mosquito cell passages that were examined.

5.3.4 Mouse Inoculation.

Twelve mouse pups from a litter of 13 which were injected with the standard inoculum developed normally until four weeks of age. One death occurred 24 hours following inoculation and was attributed to the procedure or mis-mothering. Further passages were not attempted.

5.3.5 Egg inoculation.

The chorio-allantoic membrane (CAM) from one of the eggs inoculated by that route showed a slight increase in opacity on the first passage. Histologically there was oedema and increased cellularity. A second egg inoculated with material from the first pass CAM produced no detectable gross or histological lesions. Two passages of the standard inoculum in eggs via yolk sac inoculation produced no gross or histopathological lesions.

5.4. DISCUSSION

Two types of virus-like particles (VLPs) were seen on EM. Figures 5.1 and 5.2 (~60 nm : 1.28 g/cm³) and figures 5.3 and 5.4 (~100-120 nm : 1.22 g/cm³) were candidates for the WPD virus. There is evidence that the WPD virus has an RNA genome and a lipid envelope (O'Keefe, 1996; O'Keefe *et al.*, 1997). Three RNA virus families and a "floating" RNA virus genus (arterivirus) could appear similar to the 60 nm particles observed under EM. There are no DNA viruses of vertebrates morphologically similar to the observed particles.

Togaviruses : Virions (60-70 nm) are composed of a 40 nm icosahedral nucleocapsid surrounded by a tightly adherent lipid bilayer and glycoprotein projections (Fenner *et al.*, 1993; Murphy *et al.*, 1995). Negative contrast EM of togaviruses demonstrates amorphous particles in many situations. The VLPs in figures 5.1 and 5.2 lacked distinct morphology and were considered to resemble togaviruses (Fenner *et al.*, 1993; Murphy *et al.*, 1995). Togaviruses have a wide range of buoyant densities in sucrose of (1.13-1.24 g/cm³) but relatively lower densities in CsCl (≤ 1.25 g/cm³) (Matthews, 1982).

Arteriviruses have a diameter of 60 nm, including a complex lipid envelope, and an amorphous, semi-spherical appearance on EM. A wide range of EM morphologies have been reported for arteriviruses, depending on the preparative technique, and a range of buoyant densities in both sucrose, CsCl (1.17-1.22 g/cm³) and other gradient media have been recorded (Murphy *et al.*, 1995; De Vries *et al.*, 1996).

Flaviviruses also have a size range (40-60 nm) compatible with particles 5.1 and 5.2 and a wide range of buoyant densities in sucrose (1.1-1.23 g/cm³). Densities in CsCl are not commonly reported but 1.22-1.24 g/cm³ is recorded for the type species : yellow fever virus (Murphy *et al.*, 1995). Morphologically particle 5.2 is very similar to stain penetrated hepatitis A virions demonstrated by IEM (Palmer and Martin, 1988) even though hepatitis A virus is classified within the *Picornaviridae* and has smaller overall dimensions (Hollinger and Ticehurst, 1996).

Birnaviruses are non-enveloped icosahedral RNA viruses about 60 nm in diameter (Murphy *et al.*, 1995). The VLP in figure 5.2 is shown to resemble a 60 nm icosahedron. The VLPs in figures 5.1 and 5.2 had a buoyant density of 1.28 g/cm³ in CsCl. This was not compatible with birnavirus buoyant densities of 1.33 g/cm³ in CsCl. Birnaviruses have not been previously reported to infect mammals and it was therefore unlikely (but not impossible) that WPD virus would become a new genus within the Birnaviridae.

The VLPs in figures 5.1 and 5.2 appeared similar to togaviruses and flaviviruses. Both virus families have been associated with generalised and encephalitic viral disease in humans and animals and as both were possible aetiological agents the early literature on arboviruses in N.Z. was reviewed. Serological surveys indicated arbovirus activity in south Westland and elsewhere in N.Z. (Maguire and Miles, 1960; Hogg *et al.*, 1963; Ross *et al.*, 1963). A group A arbovirus, Whataroa virus (WV), was isolated following inoculation of pooled mosquito extracts from Westland onto chick embryo monolayers (Ross *et al.*, 1963). Initial serological evidence had indicated that group B arbovirus activity was predominant in south Westland (Maguire and Miles, 1960). As there is little serological cross-reactivity between members of the group A and group B arboviruses (Calisher and Karabatsos, 1988), isolation of a group A arbovirus from south Westland should have appeared an unexpected finding. There is little mention of this apparent paradox, although Hogg *et al.*, (1963) record the possibility of one or more viruses. Available data (Dempster, 1964) showed a low overall prevalence of antibodies to WV in 76 wild caught possums and it was concluded that possums did not play a role in the ecology of WV (Maguire *et al.*, 1967). Available data suggests that the WPD agent is unlikely to be related to WV but there remains a distinct possibility that a flavivirus (former group B arboviruses) circulates undetected in Westland and perhaps in other areas of N.Z.

The buoyant density in CsCl of the VLPs in figures 5.1 and 5.2 (1.28 g/cm³) was higher than has been reported for flaviviruses and togaviruses (Matthews, 1982; Murphy *et al.*, 1995) and this would rule out these virus families for the WPD virus on strict criteria. However, both flaviviruses and togaviruses are enveloped and preferentially concentrated using sucrose solutions, or other gradient media, which are less inclined to damage viral

envelopes (Evans, 1978; Sheeler, 1981). Centrifugation in CsCl for prolonged periods may have altered the physical properties (including density) of particles exposed to this environment. Therefore, these virus families or arteriviruses should not be completely discounted.

There are several enveloped RNA virus families that infect vertebrates, that are circular or pleomorphic, that possess surface projections and are similar in size to the particles depicted in figures 5.3 and 5.4 (100-120 nm). These candidate VLPs were unlikely to be orthomyxoviruses (80-120 nm), retroviruses (80-100 nm), corona or corona-like viruses (120-160 nm) because their morphology, including that of their surface projections (figure 5.3), was not typical of these virus families.

Bunyaviruses (80-120 nm) are spherical or pleomorphic, display surface glycoprotein projections 5-10 nm in length and may resemble the VLPs seen in figures 5.3 and 5.4. Bunyaviruses have a density in CsCl of 1.20-1.21 g/cm³ and a layer of surface projections which are variably demonstrated by negative contrast EM (Murphy *et al.*, 1995). The VLPs described in figures 5.3 and 5.4 (100-120 nm), were detected at 1.22 g/cm³ and while similar to bunyaviruses in many aspects, resolution of morphological detail was relatively poor.

Arenaviruses (50-300 nm) are more typically 110-130 nm, spherical to pleomorphic and contain club-like projections from a dense lipid envelope (Murphy *et al.*, 1995). Notwithstanding the lack of sharp resolution, it appeared that the VLP in figure 5.3 had surface projections that were morphologically similar to arenaviruses. Arenavirus buoyant density is reported to be approximately 1.19-1.20 g/cm³ in CsCl (Murphy *et al.*, 1995) and the buoyant density of VLPs 5.3 and 5.4. was reasonably similar. However, the buoyant density in CsCl of the prototype arenavirus, lymphocytic choriomeningitis (LCM) virus of mice, has been reported at 1.22 g/cm³ (Lehmann-Grube, 1982) which equals the density of the gradient fraction containing VLPs 5.3 and 5.4.

Bunyaviruses cause a wide range of severe systemic diseases including encephalitis. Arenaviruses also cause clinically severe neurological disease and LCM virus infection

has been studied as a model for immune complex disease. Both the Bunyaviridae and the Arenaviridae are therefore candidate families for the WPD virus because of compatible morphology and buoyant density in CsCl.

Borna disease virus (BDV) has been associated with VLPs of two different morphologies (Zimmermann *et al.*, 1994). Particles with a buoyant density of 1.22 g/cm³ in CsCl and diameters of both 50-60 nm and 90 nm (± 10 nm) were detected following negative staining of gradient fraction preparations of BDV. Particles in figures 5.3 and 5.4 appeared to be slightly larger than 100 nm but had compatible buoyant densities. Variably sized particles up to 100 nm in diameter were also described in thin sections of BDV infected cells (Sasaki and Ludwig, 1993; Compans *et al.*, 1994). Buoyant density data and morphology, to a more limited extent, support the hypothesis that WPD may be a Borna disease-like virus. Borna disease, however, does provide an example of a viral agent that has been difficult to convincingly demonstrate and is associated with morphologically different particles.

One reason that the WPD agent was not assigned unambiguously to a virus family was the fact that candidate VLPs were very rare, making it difficult to be confident that a homogeneous population of particles was being seen. Secondly, both standard EM and IEM preparations contained a significant amount of background subcellular debris, where overlap between the buoyant densities of viruses, cell organelles and macromolecules existed (Sheeler, 1981). The preparation of three tissues for the SI meant a greater variety of confounding subcellular components than would be expected from a tissue culture or single tissue preparation.

The 'serum-in-agar' method was a direct attempt to address this problem. Clumping of viruses (IEM) that are close to their concentration limit for detection by EM can increase the sensitivity of EM by aggregating homogeneous particles (Palmer and Martin, 1988; Hayat, 1989). The EHV-1 control demonstrated that IEM resulted in aggregation of virus particles from a sample containing approximately 10⁵ TCID₅₀/ml and that individual particles had accentuated outlines. Therefore, it was not unreasonable to attempt to concentrate the WPD virus from a sample containing more than 10⁵ possum ID/ml (6.2.2) in order to exceed the threshold for detection by EM. Demonstration of

homogeneous (aggregated) populations of viruses following the incorporation of convalescent sera would add significantly to the evidence supporting visualisation (and possibly identification) of the WPD virus. One possible disadvantage of IEM is the fact that antibody molecules, in the process of adhering to target viruses, may alter the morphology of virus at the same time as accentuating it. This potential is demonstrated in figure 5.2 in which the electron lucent (hexagonal) fringe may have resulted from the a high ratio of serum to agar in the IEM procedure.

The IEM method enabled single VLPs to be identified but did not demonstrate uniform or aggregated virions indicative of a homogeneous population. This lack of hoped for success with the IEM procedure may have been due to a number of reasons. Enveloped viruses, surrounded by host-derived plasma membranes, may have been morphologically, physico-chemically (including buoyant density) and functionally (immunologically) altered by ultra-centrifugation in CsCl (Evans, 1978; Sheeler, 1981) resulting in loss of immunological properties. Another possibility was that important epitopes may have been blocked by excess antibody (hyperglobulinaemia), thereby preventing access by (presumed) WPD specific antibodies in convalescent serum. This phenomenon was demonstrated for the 'immune complex' Aleutian disease of mink (Cho and Ingram, 1972) where restoration of antigenicity of tissue preparations followed the removal of excess antibody. Reagents for the removal of antibody from enveloped viruses, where important epitopes may be associated with the lipid component of viruses, are required to preserve the viral envelope. An optimum dissociation reagent, which removes and maintains the activity of the antibody and (theoretically) the confirmation of the epitope, has been reported (Tsang and Wilkins, 1991). This neutral pH buffer has been used as a gentle method to remove antibodies from cells (Gault, pers. comm.)

Clumping of antigens (viruses) through antibody interactions relies on a narrow range of 'optimal' ratios for reactants and is enhanced by the presence of divalent or IgM classes of antibody (Klein, 1990; Feinstein, 1975). Antisera for IEM should be carefully chosen and pre-treated to ensure consistent results (Palmer and Martin, 1988). While the EHV-1 positive control demonstrated a theoretically sensitive procedure for the identification of viruses, the concentration, composition and "availability" of antibody in convalescent

possum sera was not determined. The IEM procedure was partially validated using EHV-1 but further development of the procedure for WPD is required for optimal results.

Sucrose gradients were prepared to complement CsCl gradients as sedimentation of particles is influenced by the viscosity of sucrose as well as density. Organelles, complex macromolecules and subcellular debris are separated differently and viruses band at lower relative densities (Hinton and Dobrota, 1976). It was expected that examination of WPD material prepared on sucrose gradients, theoretically a less disruptive solution, would provide further morphological information. The failure to detect VLPs was surprising and this was possibly due to antibody 'blocking' of tissue derived particles for the IEM technique (discussed above) or partial loss of the concentration advantage following volume increase with dialysis. These processes could result in a residual concentration of recognisable particles below the threshold for detection by EM.

While estimates of the size and morphology of the WPD virus, including partial characterisation, have been communicated (O'Keefe, 1996; O'Keefe *et al.*, 1997; O'Keefe pers. comm.) the WPD virus has yet to be identified and classified. Further investigation should seek to confirm our basic understanding of WPD to date. In particular, the centrifugation and EM techniques used to concentrate and demonstrate the virus respectively could be extended to a greater range of gradient media and IEM protocols. Immune-EM identification of a candidate WPD virus, with appropriate quality controls, should indicate the particles associated with the disease and give a strong approximation of their size.

It was noteworthy that all of the putative candidate families for WPD virus on morphological grounds were RNA viruses and this was in agreement with characterisation data for the presumed tissue culture isolate (O'Keefe, 1996). These complementary observations allow hypotheses on the origin of WPD to be more open because RNA viruses demonstrate a high degree of "evolvability" (Burke, 1996a). Quasi-species theories for virus evolution have suggested that RNA viruses are less confined by species boundaries than DNA viruses. Rates of mutation for RNA viruses,

due to genomic recombination, reassortments and error-prone replication are several orders of magnitude higher than equivalent processes in DNA viruses (Domingo *et al.*, 1993; Eigen, 1993; Burke, 1996a). That WPD has an RNA genome indicates a much greater chance the virus has mutated to become more virulent or cross a species boundary than would be the case if the agent of WPD had a DNA genome.

Despite intensive efforts and varied approaches WPD was not isolated *in vitro* in these studies. There are many precedents in the literature for viruses that are unable to be cultivated *in vitro*. Papillomaviruses are completely refractory to standard *in vitro* cultivation techniques (Breitburd, 1987). Many small round structured viruses, typical of the Norwalk group of viruses and relatively recently consigned to the Caliciviridae, have not been cultivated (White and Fenner, 1994d). Diagnosis of these agents relies heavily on the use of serological tests and IEM has been used extensively in this field (Kapikian *et al.*, 1996). The suspect viral aetiological agent of big liver and spleen disease of domestic fowl has yet to be simply cultivated and visualised (Ellis *et al.*, 1995). Other viruses only became cultivable after a wide range of cells and growth conditions had been trialled or more sensitive systems developed and employed to detect occult replication. Aleutian disease virus was cultivated, years after recognition of the disease, in feline cells at 31.8 °C (Pearson and Gorham, 1987) and equine infectious anaemia virus was detected following co-cultivation of peripheral blood leukocytes with an equine dermal cell line followed by immuno-fluorescent examination for viral antigen (Kobayashi and Kono, 1967). Rabbit calicivirus disease (RCD) virus has only recently, and with considerable difficulty, been propagated in rabbit hepatocyte culture (König *et al.*, 1998). While the lack of *in vitro* systems for the study and characterisation of viruses is a hindrance, research on other refractory agents has continued using satisfactory alternatives. Studies to determine the routes of transmission of WPD were able to continue using an infectious tissue suspension that was standardised prior to critical experimentation and titrated *in vivo* (chapter six).

A cytopathic agent from an infected possum was isolated in PCEFs during early studies with WPD and there is further reported evidence that this agent is associated with WPD (O'Keefe, 1996; O'Keefe *et al.*, 1997). Numerous attempts have failed to isolate the

putative agent a second time. Cultivation attempts at Massey University have progressively concentrated on the use of PCEF and primary possum cell monolayers. Lack of *in vitro* growth in these potentially sensitive, primary cells suggests that WPD virus is fastidious. However, it is feasible that cultivation in the preferred host cell has not yet been attempted or that antibodies associated with tissue inocula have routinely prevented *in vitro* infection.

As a reliable *in vitro* cultivation system would considerably aid research on WPD, and because the putative WPD virus has only been isolated once, it would be worth investing further time and effort to identify permissive cells or cell culture systems. Specialised possum cell cultures (for example leukocyte or endothelial cell) and lowered incubation temperatures would be worth investigation. Further characterisation of the WPD virus, for example pH stability, would complement transmission study data that has already been generated (chapter six). Molecular studies, especially to generate sequence information, would aid classification of WPD by identifying other viruses that could be compared with WPD.

5.5 SUMMARY

Wobbly possum disease is caused by an as yet unclassified virus. Electron microscopic examination of infectious tissue suspensions following density gradient ultracentrifugation (including the use of IEM) demonstrated few virus-like particles. Two such relatively amorphous particles (60 nm) resembled togaviruses, flaviviruses or arteriviruses and two larger particles (100-120 nm) resembled arenaviruses or bunyaviruses. Extensive attempts to propagate the causative agent *in vitro*, including the use of primary possum cells and autogenous cultures, were without success. The agent of wobbly possum disease has not been isolated in any of the systems attempted in these studies. This suggests that the agent is either so fastidious that it may never be easily cultivated or that the appropriate cells and conditions have not been provided.

Chapter 6

WOBBLY POSSUM DISEASE: ROUTES OF TRANSMISSION

6.1 INTRODUCTION

Transmission studies form an important part of any viral disease investigation because knowledge of the mechanisms of virus spread is essential for any control programme. Viruses may be shed from squamous and mucosal epithelial surfaces and be present in a range of secretions including those from the respiratory, enteric and genito-urinary tracts. Where virus is released into the environment, transmission may occur by direct contact or via contact with contaminated fomites. Viruses with a lesser tendency to be excreted, yet associated with blood or tissues can be transmitted by biting (blood feeding) arthropod vectors or iatrogenically by transplantation and transfusion procedures including needle sharing (Mims, 1982; Tyler and Fields, 1990). Systemic viral infections commonly result in the shedding of virus from a number of sites. The importance of different virus-containing secretions in the transmission of disease will depend on host factors and behaviours as well as the quantity of infectious virus shed by any particular route.

While the majority of viral infections are passed on by direct or indirect horizontal transmission, outlined above, virus infections may also be spread vertically from mother to offspring. Infection can occur across the placenta (e.g. bovine viral diarrhoea) (Thiel *et al.*, 1996), may be transmitted via contact of the newborn with an infected lower reproductive tract at parturition (e.g. herpes simplex viruses)(Whitley, 1996) or be present in milk or colostrum (e.g. caprine arthritis encephalitis)(Narayan and Cork, 1990) and passed to the newborn at suckling.

The mere presence of virus in a particular secretion is not sufficient to prove significant natural transmission by routes associated with that secretion and experimental protocols

to definitively answer hypotheses on transmission are challenging. For example, comparing two feline retroviruses shed in saliva, feline leukaemia virus (FeLV) can be transmitted by non-aggressive behaviours (allo-grooming and sharing of feed bowls)(Pedersen, 1987a) whereas feline immunodeficiency virus (FIV) is predominantly transmitted following parenteral puncture (Pedersen and Barlough, 1991; Miyazama and Mikami, 1993). Knowing the routes of transmission of a viral disease and estimations for their relative importance may explain the epidemiology of various disease entities and suggest rational controls. For example, the relatively low risk of horizontal FeLV transmission in multi-cat households may be compared to explosive foot and mouth disease epidemics in susceptible ungulate populations where virus is shed and dispersed in respiratory secretions and animals are susceptible to infection by the respiratory route. The effectiveness of condoms in reducing the rate of spread of human immuno-deficiency virus, follows the results of transmission and epidemiological studies which determined the relative risk of certain behaviours in conjunction with the presence of virus (Levy, 1993; Conant *et al.*, 1986).

The common use for transmission data is in the design of disease prevention strategies and control measures once an infectious disease has been diagnosed. Alternatively, such knowledge, together with other epidemiological data, may be used to assess the biological and environmental sustainability of an infectious disease with potential as a biological control agent or vector. It is in the latter capacity that information on the transmission of WPD was sought and the following studies conducted.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of a standard inoculum.

A standard inoculum (SI) was prepared for transmission experiments by homogenising liver, spleen and brain collected aseptically from five possums inoculated with the original Invermay infectious material and a further two possums inoculated with first passage tissues (table 4.4). Tissues from these seven possums were homogenised in MEM with a Sorvall^R Omni-mixer (Dupont Instruments), pooled and stored as a 60% suspension at -70 °C. This homogenate was thawed, diluted with an equal volume of MEM and centrifuged at 2000 x g for 15 minutes. The 170 ml of supernatant was further diluted 1:1 with MEM and passed through a glass fibre pre-filter, then through a 0.45 µm membrane, aliquotted into 4 - 4.5 ml volumes and stored at -70 °C.

6.2.2 Infectivity and titration of the standard inoculum (SI) *in vivo*.

In order to test the infectivity of the SI following storage at -70 °C, two possums (604, 605) were each inoculated with 4 ml aliquots by the intra-peritoneal (IP) route as described previously (4.2.3). When the SI was subsequently used in transmission studies, it was thawed, once only, at room temperature and held at 4 °C prior to administration which was always within three hours of thawing.

The SI was titrated *in vivo*. Serial ten-fold dilutions of the SI (10^0 to 10^{-5}) were prepared in MEM and 1 ml aliquots injected into possums by the IP route. Two juvenile possums (800 - 1700 g) were used per dilution and caged together except for the 10^{-5} dilution which was given to one adult and one juvenile possum held in separate cages.

Negative controls included a possum (660) given a clarified 10% liver suspension from a non-infected possum and two juvenile possums which did not receive inoculations (661, 680). Negative controls were held out of direct contact in separate cages but in close proximity to the inoculated possums in the same room. One of the non-inoculated controls (680) had a starting weight of 680 g and was used primarily to determine whether or not the diet was adequate for recently weaned possums. Individual possum cages and laboratory rabbit cages were used to house possums for the titration (4.2.2).

A further two possums (670, 678) received inoculations of a control SI prepared from liver, brain and spleen tissues of a non-infected possum. Tissues were prepared as 20% individual suspensions in the Sorvall^R Omni-mixer, clarified by centrifugation (2000 x g, 15 minutes) and the supernatants pooled prior to filtration (0.45 µm). Each possum received an inoculum of 5 ml by the IP route as previously described. Although these two possums were not evaluated at the same time as the titration study, they are reported here for completeness.

All possums were observed for at least 25 days unless they had developed clinical signs of WPD necessitating euthanasia for welfare reasons. All possums were necropsied to confirm whether or not they had contracted WPD. The infectivity titre, in possum infectious doses (PID₅₀), was calculated using the Karber formula (Mohanty and Dutta, 1981).

6.2.3 Transmission experiments.

Transmission experiments were conducted using similar protocols to those established for the filtration study (4.2.9). Two inoculated and one non-inoculated, control possum were held in each room. The SI was thawed and held at 4 °C for up to three hours if not used immediately. Possums were prepared and pre-inoculation sera collected (4.2.3).

Two possums (606, 607) received 4.5 ml of SI by intra-gastric placement. A 4.5 mm diameter stomach tube was passed 25 cm down the oesophagus and the inoculum deposited by syringe. Stomach tube contents were flushed through with water, the tube carefully removed and the possum held vertically for a short period to prevent reflux. A second pair of possums (612, 614) received 2 ml of SI into the trachea. One possum was shaved and swabbed with alcohol on the ventral aspect of the neck and given the SI by trans-tracheal injection through a 22 g x 5/8" needle. The second possum was intubated with the assistance of a laryngoscope and a syringe attached to a catheter (20 g x 3") used to locate and deposit the SI beyond the glottis. The third pair of possums (609, 611) was shaved at multiple sites and 2 ml of the SI inoculated, in small blebs of 100 to 200 µl, by the intradermal (ID) route.

Sentinel control possums (608, 610 and 613) in each group were housed in a separate cage placed between the inoculated possums in each room but were not sham inoculated at the time of sedation for serum collection. The standard procedure was to keep all in-contact possums alive for one month following removal of the last infected possum.

6.2.4 Infectivity in blood.

Whole blood, collected at 15 days pi from one of the clinically affected, intradermally inoculated possums (609) was injected into two possums. One possum received 4 ml by the IP route and a second received 1.5 ml in small aliquots by the ID route (as above).

Table 6.1 Transmission experiments : Inoculation of SI and blood.

Number of Possums (I.D.)	Route of Administration	Inoculum	Volume
2 (606, 607)	Intra-gastric	SI	4.5 ml
2 (612, 614)	Intra-tracheal	SI	2.0 ml
2 (609, 611)	Intra-dermal	SI	2.0 ml
1 (625)	Intra-dermal	Blood	1.5 ml
1 (626)	Intra-peritoneal	Blood	4.0 ml
3 (608, 610, 613)	Uninoculated	-	-

6.2.5 Infectivity in urine.

Urine samples collected by cystocentesis from three possums with WPD and stored at -70 °C were pooled to provide the inoculum. Pre-clinical samples were collected from two possums (654, 658) six days post- inoculation (pi) and the third sample collected from a possum (651) in the early stage of the disease at 15 days pi. The urine was centrifuged at 700 x g for 10 minutes to provide a cell free supernatant, 10 ml of which was inoculated into each of two possums (671, 676) by the IP route.

In a separate experiment, a cleanly voided, mid-stream urine sample was collected from a possum (618) that had mild clinical signs of WPD following natural exposure in the group cage an estimated two months prior to sample collection. A 12 ml aliquot of the voided urine was administered to a single recipient (631) by the IP route.

6.2.6 Presence of blood in possum faeces.

Faecal samples and the caecal contents of four possums, two inoculated with infectious tissue (674, 677) and two inoculated with control tissues (670, 678) were screened for the presence of occult blood using Haematest tablets (Ames). Briefly, a thin smear of faeces was applied to blotting paper and the tablet placed firmly on this smear. Sufficient water was placed on top of the tablet to create a droplet held *in situ* by surface tension. After 30 seconds, an additional drop of water was added with the excess allowed to run into the faeces and blotting paper. The assay was left for up to 24 hours for a colour reaction to develop. No *in vivo* assays of the infectivity of faeces were performed.

6.2.7 Infectivity in the possum itch mite.

The possum itch mite *Trichosuiolaelaps crassipes* was investigated as a potential vector for WPD. Approximately 150 mites were collected from three possums clinically affected with WPD and stored at -70 °C. Mites were thawed, pooled and disrupted in a sterile tissue grinder with approximately 800 µl of MEM containing 1% FBS and 2% PSK. An aliquot of this suspension (50 µl) was placed on top of a Haematest tablet for the detection of occult blood. The remaining 750 µl of homogenate was injected intradermally and subcutaneously into one possum (632).

In a second experiment, live mites were transferred from two infected possums (671, 673) onto a juvenile recipient (679). The mites were collected from the anaesthetised possums whilst fully mobile on the skin surface. Care was taken to preserve the integrity of the mites by allowing them to climb up the pelage and then removing the section of fur to which they were attached. Therefore, mites were handled indirectly and atraumatically and a fine, dry, artist's brush was used to aid transfer of mites to a container (held at 4 °C) for two hours prior to transfer. Approximately 50 mites were transferred on each of two occasions, five days apart, and these were observed to disperse rapidly on the skin surface of the recipient possum. The recipient possum (679) was observed for 60 days for clinical signs of WPD, euthanased and subjected to full necropsy.

6.2.8 Transmission of WPD by contact.

Seven possums were housed together uncaged in one of the temperature-controlled rooms enforcing a significant degree of cohabitation. One large wooden nest box (600 x 400 x 400 mm), one smaller wooden nest box (400 x 250 x 300 mm) and three plastic containers (220 x 220 x 400 mm) allowed for the accommodation of single possums or larger groups. Three feeding stations and watering places were provided to prevent undue competition between possums yet encourage a degree of interaction. A male (622) and a female possum (617) with a dependent pouch young were each given 4.5 ml of the standard inoculum by the IP route and released back into the room (4.2.3). The contact possums comprised one male (621) and four females (615, 616, 618 and 620), each with a back-rider or dependent pouch young, from which pre-inoculation serum samples were collected. Possums were observed daily for clinical signs of WPD and removed for necropsy either when they died or were euthanased for welfare reasons.

6.2.9 Mother to offspring transmission.

In addition to information on transmission of WPD between adults, the selection of female joeys with offspring for the 'transmission by contact' trial (6.2.8) enabled observation of maternal behaviour and whether or not transmission of WPD to young occurred. Five inoculated female possums (606, 607, 612, 614 and 626) and one non-inoculated control (613) in the individual cage transmission trials had pouch young. Whenever possible the pouch young were observed for clinical signs of WPD. Following euthanasia they were subjected to the usual necropsy examination.

6.3 RESULTS

6.3.1 Infectivity and titration of the standard inoculum (SI) *in vivo*.

Possums 604 and 605, inoculated with the SI following initial storage at -70 °C, developed clinical and histologically confirmed WPD. Clinical signs of WPD were observed in possums at all dilutions of the standard inoculum that were tested (10^0 - 10^{-5}). Possum 653 (10^{-4}) did not show any clinical signs of disease and gained weight during the trial. Possum 654 (10^0) was euthanased for welfare reasons, unrelated to WPD, at day six pi without any clinical evidence for WPD. All other possums developed clinical signs of WPD which were confirmed on histological examination (table 6.2, table 6.10). The SI was regarded as having a titre of $>10^5$ PID_{50} as both possums at the 10^{-5} dilution developed WPD. Control possums, including the smallest juvenile (680), were negative for WPD or gained weight and developed normally (table 6.13).

Table 6.2 WPD and scores for titration of standard inoculum.

INOCULATION ROUTE (Possum numbers)	DILUTION OF SI	SCORE	WPD
Intra-peritoneal (654, 659)	10^0	1, 8	1 / 1*
" " (657, 658)	10^{-1}	7, 11	2 / 2
" " (655, 656)	10^{-2}	9, 11	2 / 2
" " (650, 651)	10^{-3}	11, 7	2 / 2
" " (652, 653)	10^{-4}	8, 2	1 / 2
" " (672, 674)	10^{-5}	11, 9	2 / 2
" " (660)	normal liver	0	0 / 1
control (661, 680)	not inoculated	0, 0	0 / 2

* Possum 654 was euthanased before clinical signs were apparent (6 days pi).

6.3.2 Routes of transmission which resulted in WPD using the SI.

All possums which received the SI by the intra-gastric, intra-dermal or intra-tracheal routes became ataxic and were confirmed histologically to have WPD with scores ≥ 8 (table 6.3, table 6.11). No control animals had clinical signs or histological changes associated with WPD (table 6.2, table 6.3, table 6.13).

Table 6.3 Transmission of WPD using the standard inoculum.

INOCULATION ROUTE (Possum numbers)	INOCULATION (Volume)	SCORE	WPD
Intra-gastric (606, 607)	SI (4.5 ml)	9, 9	2 / 2
Intra-tracheal (612, 614)	SI (2.0 ml)	9, 8	2 / 2
Intra-dermal (609, 611)	SI (2.0 ml)	10, 11	2 / 2
Control (608, 610, 613)	None	1, 1, 0	0 / 3

6.3.3 Transmission of WPD with blood, urine and mite preparations.

Whole blood transferred from possum 609 (challenged by ID route) during the clinical phase of WPD established disease in two further recipients, one possum (626) following IP administration and one possum (625) after ID inoculation (table 6.4, table 6.11).

Urine : Two possums (676, 671) which received IP inoculations of acute phase urine showed early clinical signs of WPD at 7 and 10 days pi and were profoundly ataxic at 11 and 16 days pi respectively. Both possums had WPD confirmed histologically. Possum 631 that received voided urine from a naturally infected possum (618) with mild clinical signs (convalescent phase) did not develop WPD when challenged by the same route.

Homogenised mites : The mite suspension was confirmed to contain mammalian haemoglobin by a positive colour reaction to the Haematest occult blood detection system. The possum (632) inoculated by the ID route with this suspension became easy to handle and slow by 15 days pi. There was a gradual progression to ataxia and WPD was confirmed histologically following euthanasia on day 26 pi (table 6.4, table 6.11).

Mite transfer : The juvenile possum (679) that received live mites from two infected adult donors did not become ataxic or show any behavioural abnormalities. There were no histological changes in the CNS but a mild increase of inflammatory cells adjacent to some vessels in the liver and minimal interstitial nephritis was detected following euthanasia (33 days after the transfer of the first ~50 live mites). It was concluded that this recipient did not develop WPD (table 6.4, table 6.11).

Table 6.4 Transmission of WPD by urine, blood and mite preparations.

INOCULATION TYPE (Possum numbers)	INOCULATION ROUTE	SCORE	WPD
Whole blood (626)	Intra-peritoneal	9	1 / 1
Whole blood (625)	Intra-dermal	9	1 / 1
Mite homogenate (632)	Intra-dermal	10	1 / 1
Mite transfer (679)	Supra-dermal	1	0 / 1
Urine (631, 671, 676)	Intra-peritoneal	3, 9, 10	2 / 3

6.3.4 Faeces and caecal contents of possums contained occult blood.

Faeces and caecal contents of the two infected possums (674, 677) and the two control possums (670, 678) gave positive reactions for occult blood detection (Haematest).

6.3.5 Contact transmission of WPD.

All adult possums that were in contact with the two inoculated animals in the group cage became ataxic and had WPD confirmed histologically (table 6.5, table 6.12). One in-contact possum (616) died and the remaining four were euthanased when obviously clinically affected. The two inoculated possums (617, 622) showed early clinical signs of WPD at 19 and 15 days pi respectively. The contact possum which died was clinically ataxic at this stage but the remaining contact possums (615, 618, 620 and 621) were not detectably ataxic until four weeks or more following exposure to the two inoculated possums (table 6.6). All euthanased possums were in very poor body condition and two appeared moribund prior to euthanasia. Wasting in this husbandry situation appeared equal to or greater than that observed in the individual cage trials. However, pre-inoculation and post-trial bodyweights were not recorded in this experiment and no points for weight loss were allocated so that the WPD scores (table 6.5) represented minimum values.

Table 6.5 WPD scores for possums infected by natural transmission.

GROUP CAGE ADULTS (Possum numbers)	SCORE *	WPD
Contact (615, 616, 618, 620, 621)	7, 7, 8, 7, 10	5 / 5
Inoculated (617, 622)	9, 8	2 / 2

* Observations on each individual possum were difficult to make on a daily basis and the data base for possums in this experiment for behavioural indices of WPD was less comprehensive than for possums observed in individual cages. Scores represent a minimum.

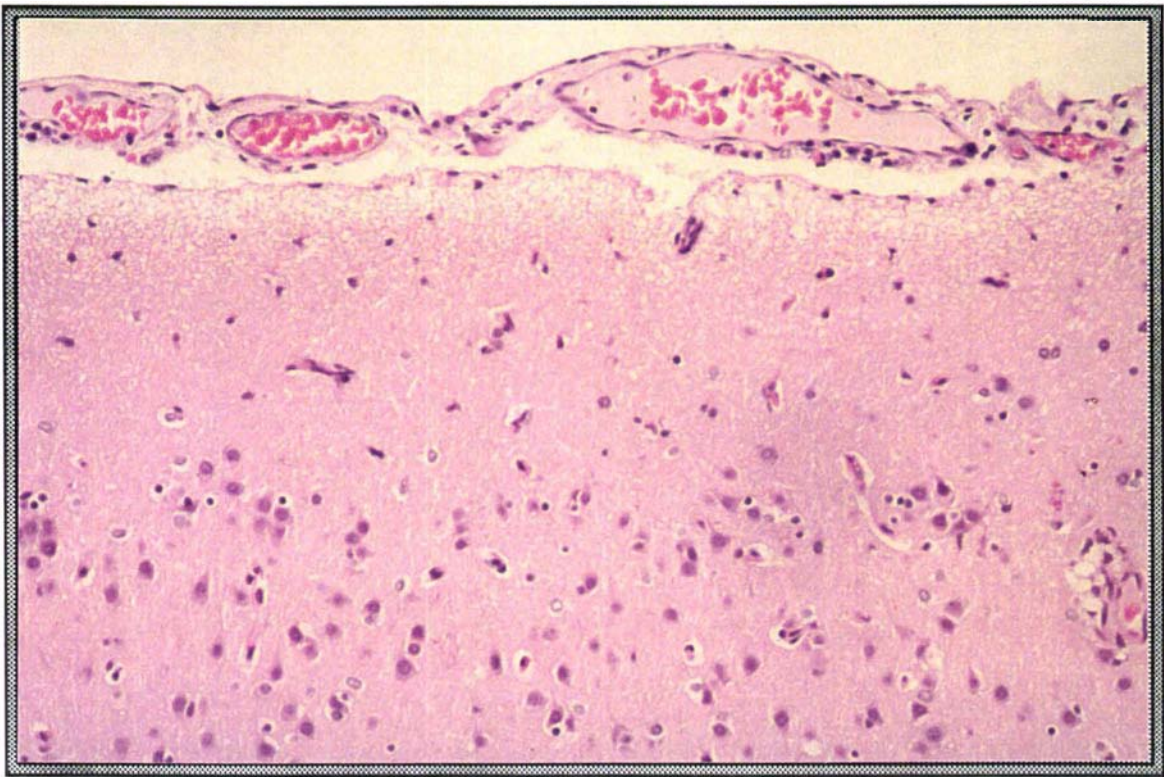
Table 6.6 Time (days) to observed behaviour changes in group cage possums.

Possum Number	Inoculated (I) Contact (C)	Behaviour change (days)	Ataxia (days)
615	C	no record	28
616	C	no record	19
617	I	no record	19
618	C	28	39
620	C	no record	32
621	C	22	29
622	I	no record	15

6.3.6 Transmission of WPD in group cage joeys.

Two of the larger joeys from the group cage environment were confirmed by clinical signs and histology to have WPD (table 6.7, table 6.12). One of these joeys (617J) belonged to an inoculated possum but became separated and was mismothered at an early stage. The second joey (620J) survived as an independent young juvenile for 43 days, including a period of 'hospitalisation' once clinical signs appeared approximately 25 days following first exposure. A third and smaller joey (616J) had mild histological lesions in the CNS (figure 6.1) which were consistent with WPD but was not observed with clinical signs and had a WPD score below the diagnostic criteria for numerical confirmation. The surviving joey (618J) from the group cage continued to be nurtured by it's mildly affected mother and showed no clinical or histological signs of WPD. The remains of one partially eaten pouch young were not investigated histologically.

Figure 6.1 Section of brain from a young possum joey 616 J ; H & E stain (x 180).



Mild, focal nonsuppurative meningitis.

Table 6.7 WPD scores for joeys infected by natural transmission.

CLASS OF CONTACT JOEY (Numbers)	SCORE	WPD
Group cage (616J ² , 617J ¹ , 618J, 620J)	3 ² , 7 ¹ , 0, 9	2 / 4
Individual cage (606J, 607J, 612J ¹ , 614J, 626J ²)	0, 0, 6 ¹ , 2 ² , 4 ²	1 / 5

1. Behavioural indices of WPD incomplete: see comment from table 6.5 above.
2. Histological evidence for WPD but no clinical signs. Six out of nine joeys had CNS lesions but only three had scores indicating WPD on numerical grounds.

6.3.7 WPD transmission in individually caged joeys.

One of five joeys from inoculated female possums used in individual cage transmission trials contracted WPD. The first signs in this possum (612J) were uneven ear carriage, ptosis, enophthalmus and prolapse of the third eyelid (Horner's syndrome). This joey was at the early 'back rider' stage and ataxia was not detected. Joey 612J had severe lesions in the CNS consistent with WPD. Another of the younger joeys (626J) had mild histological changes in the CNS consistent with WPD but no clinical signs were observed, resulting in a score less than the cut off for WPD (table 6.7, table 6.12).

For convenience of comparison, the results of all transmission trials including the control possums are summarised in tables 6.8a and 6.8b respectively.

Table 6.8a Summary of transmission data including chapter 5 experiments.

WPD in captive wild possums: various inocula by various routes and by contact.

ROUTE OF INOCULATION	TYPE OF INOCULATION	WOBBLY POSSUM DISEASE
Intra-peritoneal	WPD Tissues *	29 / 30
Intra-gastric	Tissue (SI)	2 / 2
Intra-tracheal	" "	2 / 2
Intra-dermal	" "	2 / 2
" "	Blood	1 / 1
" "	Mite homogenate	1 / 1
Mite transfer	Live mites	0 / 1 **
Intra-peritoneal	urine	2 / 3
Contact (Adult)	Not applicable	5 / 5
" " (Joey)	" "	3 / 9 ***

* Tissues for inoculation included liver, spleen, brain, blood or the SI from affected possums.

** The juvenile which received live mites had minor changes in the kidney and liver.

*** Behavioural and histological changes in joeys exposed to WPD by contact were more difficult to observe and interpret respectively than were changes in juvenile or adult possums.

Table 6.8b Control possums: Non-contact sham- or non-inoculated possums.

HUSBANDRY + PROCEDURE	TYPE OF INOCULATION	WPD
Individually caged possums	No inoculations	0 / 8
Individually caged, control possums inoculated by the IP route	Normal tissue (liver)	0 / 2
	Normal tissue (sham SI)	0 / 2
	Normal urine	0 / 1

6.3.8 Further clinical observations.

The following short paragraphs contain summary observations generated by inclusion of all transmission study possums and WPD possums described in chapter (4).

Maternal behaviour : Six female possums with severe neurological signs continued to feed and nurture dependent young. Two female possums ate their joeys and a third joey died in the pouch but was not removed by the female. Four joeys, at a back-rider stage of dependence, in the group transmission environment were observed to be unusually separated from their presumed mother. One joey (610J) was dismembered and one (joey 613J) successfully raised by female possums held in individual cages.

Convalescent period : In a few cases, possums with WPD reached a plateau beyond which clinical signs did not progress. When appetite remained adequate these possums appeared to compensate and function in spite of neurological disease. Possum 614 (housed for 6 weeks) seemed to improve and continued to nurture its dependent young, which showed no signs of WPD. Possum 618 (naturally exposed and held for more than 100 days) had mild ataxia from which an almost complete clinical recovery was made and the joey (618J) did not develop clinical disease. No other possums were kept for a period that enabled convalescence to be more than partially monitored. Several possums were assessed to be at the plateau stage when euthanased. Possums 614 and 618, clinically assessed to be in a convalescent phase, had significant and mild histological lesions consistent with WPD respectively.

Morbidity and mortality : Six adolescent or adult possums (13%) that had been inoculated and observed with clinical signs consistent with WPD died during the study (table 6.9). Two of these deaths occurred in juvenile possums during the titration trial and four deaths occurred in more adult possums given concentrated inocula. All deaths were histologically confirmed to be due to WPD. Remaining possums exposed to WPD were euthanased at a relatively advanced stage to ensure that neurological deficits were part of the clinical presentation and to facilitate histological confirmation. Wobbly possum disease was diagnosed in 44 out of 47 adult and juvenile possums (94%) that were challenged directly or indirectly with tissues or substances originating from possums with histologically confirmed WPD (table 6.9).

Table 6.9 WPD mortality in adult and juvenile possums*.

Numbers of possums	Challenge Inoculum			Route of administration	WPD (deaths)
30		tissues		IP	**29 / 30 (4)
5	tissues	blood	mites	ID	4 / 5
2		tissue		IG	2 / 2 (1)
2		tissue		IT	2 / 2
3		urine		IP	2 / 3
5		none		natural contact	5 / 5 (1)
Experimental morbidity				WPD 44 / 47 (94%)	
Mortality due to WPD				6 / 47 (13%)	

* Joeys that were diagnosed with WPD following natural exposure are not included.

** This group included 11 of the 12 possums in the titration study (chapter 6). Possum 653 (10⁻⁴) showed no signs of WPD; Possum 654 (10⁰) was not included in this analysis.

Table 6.10 Numerical classification of WPD : Infectivity and titration of the standard inoculum (SI) *in vivo*.

Possum Number	W P D Duration	Inoculum + Route	CLINICAL SIGNS					HISTOLOGICAL SIGNS					W P D Score
			Ataxia	Immobile	Behaviour	App ↓	20% Wt ↓	CNS	Liver	Kidney	Heart	Other	
604	21	SI 4 ml	+++	-	+	+	nr	++	+	+	nr	-	9
605	21	SI 4 ml	+++	-	+	-	nr	++	+	+	nr	-	8
650	24	SI 10 ⁻³ ip	+++	-	+	+	+ 20% ↓	++	+	+	+	-	11
651	15 D	SI 10 ⁻³ ip	-	-	+	+	+ 20% ↓	++	+	+	nr	-	7
652	20	SI 10 ⁻⁴ ip	+++	-	+	-	- 5% ↑	++	+	+	nr	-	8
653	26	SI 10 ⁻⁴ ip	-	-	-	-	- 11% ↑	-	+	+	-	-	2
654	6	SI 10 ⁰ ip	-	-	-	-	nr	-	+	-	-	-	1
655	19	SI 10 ⁻² ip	++	+	+	-	+ 32% ↓	++	+	+	nr	-	9
656	25	SI 10 ⁻² ip	+++	-	+	+	+ 30% ↓	++	+	+	nr	+	11
657	25	SI 10 ⁻¹ ip	-	+	-	-	+ 26% ↓	++	+	+	+	-	7
658	25	SI 10 ⁻¹ ip	++	+	+	+	+ 31% ↓	++	+	+	+	-	11
659	17 D	SI 10 ⁰ ip	+++	-	-	-	+ 24% ↓	++	+	+	-	-	8
672	21	SI 10 ⁻⁵ ip	+++	-	+	+	+ 27% ↓	++	+	+	+	-	11
674	37	SI 10 ⁻⁵ ip	++	+	-	+	+ 31% ↓	++	+	+	-	-	9

WPD Duration = Days from inoculation with WPD virus until euthanasia or death (D). Standard inoculum (SI) or dilutions by intra-peritoneal (ip) injection.

+/++/+++ = Presence of a clinical sign or histological lesion associated with WPD. Changes indicating CNS involvement are weighted (4.2.6).

App ↓ = Appetite decrease. **20% Wt ↓** = Loss of 20% of body weight during captivity / experiment. **nr** = not recorded.

Table 6.11 Numerical classification of WPD in possums challenged with the WPD virus in various ways.

Possum Number	WPD Duration	Inoculum + Route	CLINICAL SIGNS					HISTOLOGICAL SIGNS					WPD Score
			Ataxia	Immobile	Behaviour	App ↓	20% Wt ↓	CNS	Liver	Kidney	Heart	Other	
606	15 D	Gastric	++	+	+	+	nr	++	+	+	nr	-	9
607	19	Gastric	++	+	+	+	nr	++	+	+	nr	Blood ¹	9
612	21	Tracheal	+++	-	+	+	nr	++	+	+	nr	" "	9
614	42	Tracheal	++	+	-	-	nr	++	+	+	+	" "	8
609	18	Dermal	++	+	+	+	nr	++	+	+	+	-	10
611	19	Dermal	+++	+	+	+	nr	++	+	+	+	-	11
626	22	Blood ip	+++	-	+	+	nr	++	+	+	nr	-	9
625	22	Blood id	+++	-	+	+	nr	++	+	+	nr	-	9
673	20 D	Mite donor	+++	-	+	+	+ 31% ↓	++	+	+	+	-	11
632	27	Mites id	+++	-	+	+	+ 30% ↓	++	-	+	+	-	10
679	33	Live mites	-	-	-	-	Wt. gain	-	-	+	-	-	1
631	27	Urine 618	-	-	+	-	-	-	+	+	-	-	3
671	22	Urine ip	++	+	+	+	+ 24% ↓	++	+	+	+	-	11
676	21	Urine ip	+++	-	-	+	+ 25% ↓	++	+	+	-	+	10

Possums were challenged with the standard inoculum or substances potentially containing WPD virus (blood / urine / mite homogenate / live mites).

WPD Duration = Number of days from challenge with WPD virus until euthanasia or death (D). nr = not recorded.

+ / ++ / +++ = Presence of a clinical sign or histological lesion associated with WPD. Changes indicating CNS involvement were weighted (4.2.6).

App ↓ = Appetite decrease. 20% Wt ↓ = Loss of 20% of body weight during the course of captivity / experiment.

1 = Frank blood noted in faeces.

Table 6.12 Numerical classification of possums naturally infected with WPD by contact with inoculated possums.

Possum Number	WPD Duration	Inoculum + Route	CLINICAL SIGNS					HISTOLOGICAL SIGNS					WPD Score
			Ataxia	Immobile	Behaviour	App ↓	20% Wt ↓	CNS	Liver	Kidney	Heart	Other	
617	32	SI ip	+++	-	# +	# +	nr	++	+	+	+	+	≥ 9
622	28	SI ip	+++	-	# +	# +	nr	++	+	+	+	-	≥ 8
615	32	Group cage ADULT Contact	+++	-	# +	# +	# +	++	+	+	-	-	≥ 7
616	32 D		+++	-	# +	# +	# +	++	+	+	nr	-	≥ 7
618	104		+++	-	+	-	-	++	+	+	nr	-	8
620	36		++	+	# +	# -	# -	++	+	+	nr	-	≥ 7
621	29		+++	-	+	+	# +	++	+	+	nr	+	≥ 10
616J	19 D		nr	nr	nr	nr	nr	++	-	+	-	-	≥ 3
617J*	nr	JOEY	++	+	# +	# +	# +	++	+	+	nr	-	≥ 7
618J	>110		-	-	-	-	growth	-	-	-	-	-	0
620J*	43 D	Contact	++	+	+	+	nr	++	+	+	-	-	≥ 9
606J	15 D	Contact	nr	nr	nr	nr	nr	-	-	-	-	-	0
607J	15	" "	nr	nr	nr	nr	nr	-	-	nr	-	-	0
612J	21	" "	++	+	-	-	-	++	+	nr	-	-	6
614J	42	" "	nr	nr	nr	nr	nr	++	-	-	nr	-	≥ 2
626J	22	" "	nr	nr	nr	nr	nr	++	+	+	nr	-	≥ 4

WPD Duration = Number of days from exposure to wobbly possum disease virus, following close contact with inoculated possums, until euthanasia or death (D).

+/++/+++ = Presence of a clinical sign or histological lesion associated with WPD. Changes indicating CNS involvement are weighted (4.2.6).

App ↓ = Appetite decrease. **20% Wt ↓** = Loss of 20% of body weight during captivity / experiment. **nr** = not recorded. **SI** = Standard inoculum

***** = Joey 617 and 620 were orphaned and parentage was deduced from earlier observation. Joey 620 was mature enough to be independent.

= Possum observations from the group cage. Records in this database were less complete than equivalent records from individually caged possums.

Table 6.13 Numerical classification of WPD in possums used as controls.

Possum Number	Exposure Duration	Inoculum + Route	CLINICAL SIGNS					HISTOLOGICAL SIGNS				WPD Score
			Ataxia	Immobile	Behaviour	App ↓	20% Wt ↓	CNS	Liver	Kidney	Heart	
608	35	Control	-	-	-	-	nr	-	-	-	+	1
610	35	Control	-	-	+	-	nr	-	-	-	-	1
613	42	Control	-	-	-	-	nr	-	-	-	-	0
613J	42	Control	-	-	-	-	nr	-	-	+	-	1
660	27	Liver	-	-	+	-	no change	-	-	-	-	1
661	28	Control	-	-	-	-	gain	-	-	-	-	0
670	36	Liver, brain and spleen	-	-	-	-	gain	-	fatty	-	-	0
678	36		-	-	-	-	gain	-	fatty	-	-	0
680	35	Control (diet)	-	-	-	-	gain	-	fatty	-	-	0

Control possums were not inoculated or were inoculated with tissues (liver, brain and spleen) from clinically normal possums by the intra-peritoneal route.

All control possums were caged individually and separately from possums inoculated with WPD virus containing tissues or directly exposed to potentially infectious substances. Cages housing control possums were placed closely adjacent to cages containing possums challenged with WPD.

Exposure duration = Number of days from initial exposure to possums challenged with infectious WPD virus until euthanasia.

Whenever possible, control possums were kept for three to four weeks following the euthanasia or death of WPD possums.

App ↓ = Appetite decrease. 20% Wt ↓ = Loss of 20% of body weight during the course of captivity / experiment.

+ = Presence of a clinical sign or histological lesion associated with WPD. nr = not recorded.

6.4 DISCUSSION

The aim of the transmission trials was to determine by which routes possums were susceptible to infection with the WPD agent and whether blood or urine were infectious substances. Although these experiments were somewhat contrived, they were a necessary first step from which more refined and biologically feasible trials could be designed. When it appeared that transmission of WPD required close contact between possums an experiment to observe the natural spread of WPD, albeit in experimental facilities, allowed an opportunity to confirm the transmissibility of the disease and speculate on likely mechanisms of transmission.

Intra-tracheal transmission : Although both possums inoculated by the intra-tracheal route became affected with WPD, no respiratory signs were observed. Ocular or nasal discharges were very infrequently observed in possums inoculated or exposed to WPD by any route and only for short durations compared to other signs. Sneezing was almost never heard in the possum rooms. Upper respiratory tract (URT) signs did not form part of the clinical picture for WPD and nor did it appear that aerosols for droplet spread of WPD were created. Control possums caged separately but adjacent to inoculated possums did not develop WPD. As the distance between cages was as little as 10-20 cm it was considered unlikely that WPD was naturally transmitted by aerosol. If the respiratory route contributes to the spread of WPD it is likely to be in the context of direct contact between contagion and the URT following possum interactions or exposure to fomites.

When the SI was subsequently shown to contain in excess of 10^5 PID₅₀ per ml it was considered probable that natural exposure, if it were to occur by this route, would be with much less virus. Refinements of challenge protocols for further investigation of URT routes of transfer might include intra-nasal inoculation of possums with an aerosol preparation containing fewer infectious doses.

Intra-gastric transmission : The establishment of WPD following intra-gastric (IG) placement of the SI suggested that the virus was able to survive a period of transit through the possum's upper gastro-intestinal tract (GIT). It is therefore possible for WPD to be transmitted by the oral route and this may be a significant means of natural

infection. As with intra-tracheal challenge, it was recognised that a large infectious dose had been administered ($> 4 \times 10^5$ PID) and that the bypassing of the upper GIT by a 1/10,000 or less of the challenge dose (possibly protected by SI tissue constituents or ingesta) may have been sufficient to establish WPD. This could have occurred if the SI was placed close to the duodenum in sedated possums. In either case, production of a fatal infection indicates the ability of WPD to be uptaken from the GIT. Alternatively, epithelial trauma to the oesophageal or gastric mucosae may have allowed penetration at these sites when this would not occur with natural exposure. Further work is needed to confirm the oral route of transmission for WPD. For example, possums could be challenged by allowing them to eat food which had been deliberately contaminated with the WPD agent.

Intra-dermal transmission : The ID route was demonstrated to be a biologically feasible means for the transmission of WPD. Not only was blood infectious by this route but, in the experiment in which mites from infected possums were injected intra-dermally, it was calculated that as few as 100 PID_{50} were sufficient to establish WPD by this route. [The volume of the gut of *Trichosuro laelaps crassipes* has been calculated to be approximately 7×10^{-6} ml (Clark, 1995b). If it is assumed that blood contains a similar number of infectious particles as the SI, a simple calculation puts the dose administered with 150 homogenised mites at about 100 PID_{50} .]

These results indicate that a number of direct possum/possum interactions could potentially result in virus spread. Agonistic encounters arising from territorial disputes or during the mating season could facilitate blood and tissue fluids being passed between possums. The transmission experiment with live mites, which were handled indirectly (6.2.7) and unlikely to have been damaged in the transfer, failed to establish WPD. The single recipient possum (679) had no behavioural signs of WPD and no lesions in the CNS. However, there were very mild histological changes in the liver and kidney and it is possible that this possum may have had an asymptomatic infection with the WPD agent. Currently, there is insufficient data to conclude a significant role for the possum mite in the transmission of WPD and live mite transfer (including an obligatory minimal transfer of possum blood on mite mouth parts) is unlikely to be a major mechanism for WPD dispersal in the environment. However, there are many assumptions upon which such an argument rests and, with only one recipient possum in the transfer experiment,

a role for the possum itch mite, *Trichosuiolaelaps crassipes*, in the transmission of WPD can not be ruled out. The experiment demonstrating WPD transmission following ID injection of mites did establish, without any ambiguity, that possums are relatively susceptible to infection by this route.

While the life cycle of *Trichosuiolaelaps crassipes* is completed on one host, opportunity for horizontal transmission occurs during possum mating, mother/offspring contact and den sharing. Recorded possum behaviour including allo-grooming, recorded in experimental (Day, 1996) and in natural settings (MacLennan, 1984), allows ample opportunity for mites to obtain new hosts. Infection of possums with WPD is also conceivable following ingestion of infected mites. Possum blood contained within the mite exoskeleton could bypass potential inactivation in the stomach and this consequence has been considered in the context of intra-gastric transmission above. Discussion of this potential route of transmission has been presented (Hudson *et al.*, 1995) as 'consumption of vectors' in transmission of louping ill to grouse following ingestion of infected ticks (Hudson, unpublished data). Further discussion of the life cycle and potential for the possum itch mite to act as a disease vector is presented in two recent articles (Clark 1995a, Clark 1995b).

Pseudo-vertical transmission : A numerical diagnosis of WPD was made in three joeys out of nine (33%) exposed to the WPD agent and five out of nine (56%) had CNS lesions consistent with WPD. Young possums which contracted WPD following contact with an infected dam may have been infected by a variety of routes as the intimate contact between female and pouch offspring allows ample opportunity for the agent to be transferred from maternal or environmental sources in the process of caring for young. In addition to infection by oral or intradermal mechanisms (discussed above), infection following ingestion of milk is possible if the virus was present in mammary gland tissue and had entered secretions. For example, transmission of louping ill (LI) virus to suckling kids has been reported following the infection of female goats (Reid *et al.*, 1984).

In order to detect infectious virus in milk a straight forward *in vivo* transmission experiment by IP inoculation could be performed. To determine whether or not infection

can be transmitted in milk, oral challenge, with milk from infected possums, to dependent joeys from non-infected females would be necessary in order to avoid background horizontal transmission. Difficulty was encountered with assessment of the behavioural indices of WPD in joeys and with differentiation of mild histological lesions from non-specific histological changes. Therefore, the scoring system was not ideal for determining the outcome of WPD exposure in this youngest class of possum. If the severity of WPD and hypothesised mortality of female possums in the wild is similar to the experimental disease, few dependent joeys would be expected to survive. Consequently, further investigation of WPD transfer to offspring was not contemplated.

Environmental contamination : Urine, during the acute phase of WPD, is infectious and could thus act as a source of environmental contamination. Possums inoculated with acute phase urine were rapidly and severely affected with WPD and this was consistent with the observation of lesions in the kidneys of affected possums. Convalescent urine from a possum, confirmed to have WPD and considered to have been improving when the sample was obtained, did not produce WPD. This suggested there were no infectious virus particles in the challenge dose and that mechanisms (viraemia or local replication) resulting in the presence of virus in the urine were limited in time. Alternatively, a chronic infection and low level virus excretion from the urine donor may have resulted in subclinical infection in the recipient.

The presence of occult blood in faecal and caecal samples of both infected and uninfected possums suggests that, as blood contains the WPD agent, faeces may provide another source of contamination for the environment. It is possible that WPD virus may gain access to the GIT via the biliary system following replication in the liver. This route of virus excretion has been demonstrated in rabbit calicivirus disease (Westbury *et al.*, 1994) and is feasible for non-enveloped viruses in particular but, because bile disrupts the integrity of viral envelopes, it is unlikely to be a route for enveloped viruses.

The demonstrated infectivity of urine and hypothesised infectivity of faeces indicates considerable potential for possums to pass infection on via the environment. The social organisation of possums has been described as a system of mutual avoidance between co-dominants (Cowan, 1990) and only areas adjacent to dens are actively defended. Scent

marking is postulated to communicate important information on social status, gender, age and proximity and secretions from perianal glands can be actively deposited alone or with urine (Biggins, 1984). Therefore, urine containing secretions are strategically deposited in the environment and may also be closely investigated. Urine and faecal contamination of den sites has been frequently observed and this localised increase of environmental contamination could transfer infection between individual possums using the same site on different occasions. It is speculated that infection could be acquired by ingestion from the environment, by percutaneous contamination of injuries and abrasions sustained elsewhere or by routes which have not yet been demonstrated to transmit disease (e.g. the nasal mucosa and pharynx).

Possum denning behaviour has been studied in different environments and in the context of transmission of *Mycobacterium bovis* (Fairweather *et al.*, 1986; Green and Coleman, 1987; Cowan, 1989; Paterson *et al.*, 1995; Caley, 1996). Den location and denning behaviour varies according to habitat but a common finding was the frequent use of dens by different possums (most commonly on separate occasions). The recorded tendency (Cowan, 1990; Cowan unpublished observations) for possums to travel between and favour certain den sites, provides an opportunity for the spread of WPD between possums sharing a den site either simultaneously or consecutively. Periods of poor weather decrease the number of adequate den sites and increase both the amount of sharing and time spent in the den. The frequency of den sharing by pairs is increased in the mating season. Therefore, direct possum interactions (fighting; close proximity aggressive calling; mating; allo-grooming) (Day, 1996), many of which only occur in the context of defending or sharing den sites, provide many potential mechanisms for WPD to be transmitted in nature.

Direct transfer between possums : Control possums caged in proximity to, but in no direct contact with, inoculated possums never developed WPD. In contrast, direct contact resulted in the development of WPD in non-inoculated possums in the group cage. Both adult possums and dependent offspring became affected with WPD but the disease was more difficult to confirm in young possums. Only one immature possum in the group cage remained completely free of clinical signs and developed normally. The mechanisms by which the agent is naturally transferred are unknown. No sexual activity

was recorded and observed aggressive interactions were limited to posturing and strong vocalisation. There was little fur missing to suggest actual physical encounters in the group cage and no traumatic injuries attributable to fighting were recorded. Sharing of the main den site (a large wooden box) was frequently observed and this area, visibly contaminated with urine and faeces, required regular cleaning. Any of the direct possum interactions described above and/or contact with a contaminated environment could have led to the transfer of WPD between possums in the group cage. From the limited observations of possum behaviour in this setting, it appeared that crowding behaviour and a constantly contaminated environment were more likely to have resulted in transfer of WPD between possums than agonistic behaviour which was infrequently recorded.

In addition to refinements of experiments already performed and discussed, two other biologically feasible mechanisms of transmission were not specifically addressed in this study. Saliva could be assayed for the presence of infectious virus by IP (or ID) challenge of naïve possums and this would determine a potential role for biting, allo-grooming or feed sharing (frequently observed in captive possums) in the transmission of WPD. Saliva, if demonstrated to contain virus, has considerable potential to be deposited in bite wounds, abrasions or punctures, in amounts potentially capable of transmitting infection.

A more demanding set of experiments would be necessary to determine if venereal transmission has a place in the spread of WPD. Sexual transmission of WPD may occur through increased social contact in a relatively solitary species and courtship behaviours may result in minor injuries and the transfer of infectious fluids or secretions. Venereal transmission refers to the potential for copulation to result in transfer of infection. Semen, uterine fluids and secretions from accessory sexual glands could be assayed for viral infectivity in the same manner that established the infectivity of urine. In addition, possums could be challenged with the standard inoculum or appropriate secretions by cloacal and/or vaginal placement in male and female possums respectively. Possums have a common urogenital opening in which urine, faeces and reproductive fluids have contact. During copulation, even in the absence of virus in reproductive secretions, an uninfected possum is at risk from residues of urine and/or faeces in the cloaca. Mating itself can result in trauma to mucosal epithelia, making this both a potential way of increasing

contamination of the mating environment and providing an entry point for the virus to infect a naïve host. It is likely that sexual transmission of WPD will play a role in the spread of this disease as the associated behaviours bring possums into close contact. If the venereal route also transmits WPD it is unlikely to be the sole means of transfer and it's contribution to the spread of WPD may be difficult to calculate.

As blood has been shown to be infectious, WPD virus is widely distributed in possum tissues. This could be due to a viraemic phase of unknown duration prior to localisation in a target tissue(s). Alternatively, WPD virus may replicate and disseminate from a number of different tissues or be widely distributed due to replication in a circulating host blood cell or in vascular endothelial cells. Therefore, WPD virus may be present in a greater variety of secretions and substances than have been demonstrated above and likewise, possums may be susceptible to infection by more routes than have been shown to date. It is difficult for transmission experiments alone to determine the exact route(s) of infection. For example, although possums were susceptible to WPD by the gastric route, natural infection may be established in the pharynx or adjacent tissues first. Infectious substances do not invariably remain where they are deposited or cause infection at that site, making conclusions from certain forms of inoculation experiments tentative.

Environmental contamination with WPD by urine and probably faeces may be sufficient to spread WPD if possum density is high and the agent is stable in the environment (no data on the survival of WPD virus in the environment is currently available). It is possible that the predominant mode or route of transmission of WPD may vary as a function of the possum population density. In an area of high population density, environmental contamination and transfer through conditions of crowding (ingestion, percutaneous exposure) may operate in a manner analogous to the contrived experimental situation discussed above. In support of this hypothesis we note that during the possum survey (chapter 3), when available den sites were in excess of possum numbers, some possums would shelter on top of other dead and decomposing individuals. This type of behaviour has also been recorded in 'natural' environments (Fairweather *et al.*, 1986; Cowan pers. comm.).

In contrast, in areas of low population density, sexual transmission, in its widest context, may be the major reason for possum interactions of any kind and possum encounters would be of greater importance than environmental exposure. Full characterisation of the virus and further transmission experiments are necessary in order to develop hypotheses on the mechanism(s) of transmission and epidemiology of WPD.

It is difficult to extrapolate from the morbidity and mortality data generated during these investigations to a natural situation. Morbidity data is biased by the unnatural route and form of many of the inoculation protocols. For example, natural challenge via the respiratory route would not be in the form of intra-tracheal tissue deposition. Furthermore, it could not be confirmed that challenge materials, not resulting in WPD, actually contained the infectious agent (10^{-4} dilution of SI, live mite transfer and "convalescent urine"). Many possums were humanely killed when WPD appeared advanced and the observed death rate was almost certainly an under-estimate of the true mortality of experimental WPD. Artificial husbandry and captivity stress may also have affected certain aspects of the pathogenesis of WPD. However, possums were adequately fed and housed and to some extent protected from the requirement to find shelter and forage effectively when severely affected by WPD.

In spite of the limitations of some transmission experiments (discussed above), several investigations demonstrated possum susceptibility to the WPD agent and these included the titration challenges with 10^{-4} and 10^{-5} PID_{50} , the challenge with homogenised mites, the challenge with acute phase urine and the group cage transmission experiment. The results of preliminary transmission experiments demonstrate that WPD is very frequently a severe, fatally infectious disease capable of being spread by close contact between possums.

6.5 SUMMARY

Tissues from possums infected with WPD were collected and used to prepare a standard inoculum (SI) containing greater than 10^5 PID₅₀ / ml. Inoculation of possums with the SI by the IG, IT and ID routes resulted in WPD. Control possums, housed closely adjacent to infected possums, did not develop WPD and no inoculated possums developed respiratory signs of disease. Thus, WPD is not considered to be spread by aerosol. In contrast, WPD was efficiently transmitted to all non-inoculated adult possums and at least three out of nine joeys following direct contact with inoculated possums.

Blood from infected possums transmitted WPD to possums inoculated by the IP and ID routes. Therefore, WPD virus is widely distributed in possum tissues and ID transmission, associated with antisocial behaviours and mating interactions, is a biologically feasible means for WPD virus to be transferred between possums. Transfer of live mites from possums infected with WPD did not result in transmission of disease to a recipient possum. However, when a homogenised suspension of mites, recovered from WPD infected possums, did result in the transmission of WPD to a recipient it was presumed to be due to possum blood contained in the inoculum. A role for the possum itch mite (*Trichosuirolaelaps crassipes*) was not definitively ruled out.

Urine was demonstrated to contain infectious virus. Possum faeces were demonstrated to contain occult blood. The mechanism(s) of WPD transfer have not been fully elucidated but urine and faeces are considered to be the most likely substances contaminating the environment. Wobbly possum disease virus has considerable opportunity to gain access to pre-existing or recently acquired wounds in a contaminated environment and eating and grooming behaviour where contamination is present would ensure contact between the virus, the oral cavity and the external nares.

Although further critical experimentation is required to determine by which route(s) the possum is most susceptible to infection and whether or not direct possum to possum contact is necessary for spread, WPD is a readily transmitted and fatal disease in an experimental setting. Wobbly possum disease is worth consideration as a biological control agent or vector for immunocontraception strategies on the basis of these preliminary transmission study findings.

Chapter 7

INVESTIGATION OF A DISEASE SYNDROME IN A FREE LIVING POSSUM POPULATION.

7.1 INTRODUCTION

Possums from sources other than the survey were thoroughly investigated when history, clinical examination or necropsy findings indicated a possible viral aetiology. Members of the public and the N.Z. scientific community were asked to notify any unusual changes seen in possums either within a research establishment or in the wild. One investigation of disease in a wild possum population occurred in July 1994, when sightings of sick possums in the Rotorua district were reported by an interested member of the public. Possums were reported in poor condition with wetness around the perineum, attributed to scouring, and feeding or aimless wandering during daylight hours. These possums were often able to be approached and individuals with these signs or considered to be affected at an early stage of the disease process were initially submitted for investigation to the Ruakura Animal Health Laboratory (RAHL).

7.2 MATERIALS AND METHODS

7.2.1 Field investigation.

The property where sightings of sick possums had been made lay between lake Rotoiti and the Bay of Plenty and was bounded by Rotoehu Road to the South and Maniatutu Road to the West. The property was minimally improved and large areas were covered in regrown native vegetation. A few emergent podocarps remained from the original forest (rimu and totara) but most vegetation was of mixed broadleaf and manuka species. The topography of the area was that of a moderately dissected, low volcanic plain. Improved pastures were found on the plains and in the flatter water courses. Slopes of the farm investigated were invariably covered with regrowth vegetation but many freehold farms in the area were predominantly clear. Land in neighbouring areas, administered by the crown for forestry use or held by local Maori, was often unimproved.

Possums were sighted on numerous occasions during a night expedition to the property and surrounding area and although three were captured (R002, R003 and R004), none had neurological signs. No possums were sighted during a late afternoon expedition. An affected possum (R001), which had been previously captured by the property owner, showed no inclination to escape from an incompletely covered cardboard box and very little interactive behaviour. It was able to move extremely slowly when out of the box but was timid and lacking in exploratory behaviours. Appetite was low, condition moderate to low and voidance of urine and faeces normal.

Several neighbouring farmers reported sightings of possums during the daytime and some of these animals were described as unusually complacent with respect to investigation by dogs. No affected possums were submitted from neighbouring farms.

The three apparently normal possums (R002, R003 and R004) caught during the night search and the clinically affected possum (R001) were transported to Massey University and housed at SAPU in the group enclosure (3.2.3). All possums were fed in the manner previously described and held, in the same group cage, with two resident possums that were part of the survey of normal possums. Possums subsequently observed with signs fitting the syndrome were couriered to Massey. Possum R005 was captured unharmed and submitted live. Possum R006 was killed by dogs and submitted as a chilled carcass. Possum R007 was submitted as a frozen carcass.

Observations on possums R001 - R004 were initially made by SAPU personnel and untoward findings reported. When deaths occurred, observations prior to death were recorded and the time between death and post mortem estimated from history, clinical appearance (rigor) and the post mortem examination. Possums were subjected to a full necropsy and samples were taken for microbiology. Tissues were collected for virology and stored at -70 °C or used in explant or co-cultivation tissue culture assays. Tissue samples were also collected into 10% neutral buffered formalin and processed for histopathological examination.

7.2.2 Tissue culture.

R001 - Samples of kidney, lung, lymph node, salivary gland, adrenal gland, bladder, prostate and tonsil were collected aseptically and processed as primary explant cultures as previously described (Pye *et al.*, 1977) (3.2.7). The enriched growth medium (appendix 1) was changed twice weekly once outgrowth was apparent. Confluent primary monolayers were passaged. When outgrowth was judged to be at a maximum and/or deterioration of the explant islands or monolayer was detected, the multiplate was frozen and stored at -70°C. The multiplates were subsequently frozen and thawed twice and aliquots of these primary cell cultures passaged three times on OPK, PTK2 and PRT-1/2 cells. At the conclusion of the third pass, monolayers were tested for haemadsorption and examined cytologically (3.2.6, figure 3.2)

R003 - Samples of kidney, salivary gland, lung, lymph node and prostate were processed as for R001.

R005 - This possum was electively euthanased and samples of kidney, salivary gland, lung, bladder and tonsil tissues processed as for R001. In addition, co-cultivation of trigeminal ganglia, dorsal root ganglia, brain stem, tonsil, heart and buffy coat cells was performed (explant primary culture passage 3.2.8).

Virus isolation was not attempted from possums **R002**, **R004**, **R006** and **R007**.

7.2.3 Transmission trials.

All possums challenged with tissues from the original Rotorua possums, or with tissues subsequently passaged in possums, were captured in the Manawatu region and appeared clinically normal prior to inoculation. An extended period of pre-inoculation observation and adjustment to the experimental facilities was not feasible for most of the possums used in these challenge experiments. Possum maintenance and handling procedures, including sedation, blood collection, preparation for inoculation and euthanasia, have been previously described (chapter 3).

(i) Experiment one :

Fresh liver and spleen tissue (approximately 1 cm³ total) from R001 (stored at -70 °C) was thawed and disrupted in a Tenbroek tissue grinder with 9 ml MEM to prepare a 10% suspension. The suspension was lightly clarified by centrifugation (200 x g) for ten minutes and 4.5 ml of supernatant stored for one hour at 4 °C prior to IP injection into a female possum (623). The remaining suspension was centrifuged (2700 x g) for 30 minutes and the clarified supernatant was filtered through a 0.45 µm membrane. The filtered supernatant (4.5 ml) was injected into a second female possum (624) by the IP route. Laboratory rabbit cages (4.2.2) that had previously housed possums for the WPD titration experiment (6.2.2) were cleaned and disinfected with Virkon™ and fumigated with formalin vapour prior to housing possums 623 and 624. The possums were kept in a ground floor room separated by seven floors from the area where the WPD studies were conducted. Separate food supplies, litter and cleaning implements were maintained. Cleaning, feeding and observation of the possums was undertaken at the start of the day or by personnel who had not had contact with other possums on that day. A fresh set of autoclaved protective clothing and disposable gloves were worn.

(ii) Experiment two :

Liver and spleen tissue (1 cm³ total) from R001 was thawed for a second time, minced and disrupted in a Tenbroek tissue grinder (20% suspension in MEM containing 1% FBS and 2% PSK), centrifuged (700 x g for 10 minutes) and 4 ml injected into one female possum (639) by the IP route. A pooled brain and spleen sample (1 cm³ tissue), also from possum R001, was prepared in an identical manner and 4 ml injected into a male possum (638) by the IP route. A female possum (635) that did not receive an inoculum was placed in a separate, adjacent cage.

(iii) Experiment three :

Tissues from possums 623 and 624 (experiment one) were collected aseptically at euthanasia. Half the brain, an equivalent volume of liver and the spleen from each possum was pooled and homogenised to form 20% suspensions (Sorvall^R Omni-mixer, Dupont Instruments) in MEM (containing 1% FBS) at 4 °C. Suspensions were frozen and thawed twice and centrifuged (2700 x g for 30 minutes). Half of the supernatant

from each suspension was filtered (0.45 μ m) and 4.0 ml aliquots stored at -70 °C. Fungizone (2%)(Gibco, BRL) and PSK (2%) were added to the remaining supernatants which were then stored as above. Second passage inoculations were made by the IP route into two male possums. Possum 634 received 4 ml of filtered supernatant derived from pooled tissues of possum 623 while possum 633 received 4 ml of unfiltered supernatant derived from pooled tissues of possum 624.

Possums 633 and 634 were sedated 5, 8, 13 and 19 days following IP inoculation with infectious liver, spleen and brain suspensions and 3 - 5 ml of blood collected by jugular venepuncture into EDTA vacutainers. The blood was centrifuged at 2000 x g and the buffy coat aspirated. Contaminating RBC were lysed by addition of five volumes of filtered dH₂O for 45 seconds. Osmolarity was restored with an equal volume of 0.3 M NaCl and WBCs recovered by centrifugation (400 x g). Separate preparations of whole blood and the enriched WBC-containing sample were inoculated onto PCEF monolayers in small flasks (3.2.7). The same samples (whole blood and WBC) were duplicated on PCEF monolayers that were recently established (60-80% confluent). The four culture variations per blood sample were pooled at the end of the first passage, frozen and thawed 2-3 times and passed onto fresh PCEFs. Seven additional passes were performed using sub-confluent PCEF monolayers.

The five possums (633, 634, 638, 639 and 635) in experiments two and three were housed in individual possum cages described in 4.2.2 and these were located in a vacant room at SAPU. The cages were thoroughly sprayed and washed with Virkon™ prior to use and the room had never previously housed possums. Separate feed supplies were maintained, autoclaved overalls and disposable gloves were worn for all handling procedures and personnel that attended these possums did not have contact with possums inoculated with WPD on that day.

(iv) Experiment four :

The liver, brain and spleen tissues from possum 638 (experiment two) were collected aseptically at elective necropsy 22 days after inoculation. Individual 20% homogenised suspensions of these tissues were prepared (described above) and centrifuged at 2700 x g for 30 minutes. The suspensions were frozen and thawed, centrifuged a second time

(2700 x g for 30 minutes) and pooled to prepare a 4 ml aliquot for inoculation into two possums (675 and 677) by the IP route. A pooled, 20% inoculum of liver, brain and spleen was prepared from a normal wild-caught possum from the Manawatu area. Four mls was injected IP into possum 678. Possums used in this trial were kept on floor eight of the Veterinary Faculty at Massey University in one of the rooms used previously to house possums challenged with WPD. The room and all cages used had been sprayed with Virkon™ and spelled for one month. Barrier nursing protocols (4.2.9) were instituted to prevent inadvertent exposure to WPD. The most relevant details of the four transmission experiments, including results, are summarised in table 7.1.

7.2.4 Observations, analyses and comparisons.

Possums were examined daily and behavioural changes and clinical signs recorded. All possums in the four experiments were euthanased when clinical signs were apparent or by 36 days post-inoculation (pi) and subjected to post-mortem examination including histopathology. The time to 'onset of behaviour change' and to 'onset of ataxia' in possums challenged either with tissue suspensions from possums with WPD or with the Rotorua syndrome were statistically analysed. The means of the two sample populations were compared using a two tailed *t* - test (GraphPad Prism™ version 2.01, GraphPad Software, Inc. San Diego). The onset of these changes was timed from a point in the daily records when observations indicating behaviour change or ataxia were recorded with confidence. While allowing for differences in observer comments, an attempt to be consistent across all records was made.

Recuts of paraffin blocked tissues from confirmed cases of WPD and the Rotorua syndrome were appropriately prepared and randomly identified by technical staff. "Blinded" sets of tissues were examined and second opinions were kindly provided by Ms Michèle M. Cooke and Professor B.W. Manktelow. The scoring system for WPD was used to collate histopathological changes and clinical signs for each Rotorua possum investigated and each possum inoculated with tissues derived from possum R001.

Table 7.1 Summary of Rotorua syndrome experimental passages and results.

Original Case	FIRST PASSAGE			SECOND PASSAGE		
	Tissue Inoculum	Possum Number	Results () Days to ataxia or death	Tissue Inoculum	Possum Number	Results () Days to ataxia or death
R001*	Spleen / Liver	623*	Ataxia (16), mild meningo-encephalitis. Interstitial nephritis, hepatitis and myocarditis. **	Liver / spleen / brain Filtered 0.45µm.	634	Depression (9 - 11) and death (21). Cause of death not determined. No ataxia or lesions typical of WPD.
	Spleen / Liver Filtered 0.45µm.	624*	Ataxia (16), mild meningo-encephalitis. Interstitial nephritis and myocarditis. **	Liver / spleen / brain	633*	Mild behaviour changes (12) and slow movement. Meningeal congestion. Hepatitis and nephritis. **
	Spleen / Liver	639	No behaviour change. No ataxia. Localised CNS lesion seen. Hepatitis and nephritis. **	(No ongoing passage)		
	Brain / Spleen	638*	Very ataxic (17), mild meningo-encephalitis. Hepatitis, nephritis and myocarditis. **	Liver / spleen / brain	675*	Depression, inco-ordination (13) and ataxia (21). Mild meningitis, hepatitis and interstitial nephritis. **
					677*	Long incubation. Behaviour change (17), ataxia (22). Mild meningitis. Hepatitis, nephritis and myocarditis. **

* Possums that were characterised as having WPD using numerical criteria developed for WPD in chapter four (Table 7.3).

** Many histological lesions in possums affected with the Rotorua syndrome were similar or identical to lesions seen with WPD.

7.3 RESULTS

7.3.1 Earlier cases examined at Ruakura Animal Health Laboratory.

The details of investigations of four possums which had been earlier submitted to MAF Rotorua with a suspected infectious disease and forwarded to RAHL were supplied. These possums were subjected to a full necropsy and samples for histology, virology and bacteriology were collected. Reported histological lesions included :

1. Multiple foci of lymphocytic infiltration in the kidney cortex and multiple focal areas of fibrosis containing plasma cells within the myocardium (one possum).
2. Mild mononuclear cell infiltration in portal areas of the liver and eosinophilic intra-nuclear inclusions in hepatocytes (one possum).
3. Mild mononuclear cell, perivascular cuffing of a few small vessels in the brain. Mild lesions were also seen in the liver, heart and kidney as above (one possum).
4. A tubular adenocarcinoma, adjacent to facial skeletal muscle, that may have been of salivary gland tissue origin was considered to be an incidental finding (one possum).

Virus isolation was attempted from three possums with negative results but a coagulase negative staphylococcus was isolated from lung tissue of the second possum. No common pattern of lesions was discerned in the submitted possums (Fairley, pers. comm.) and further investigations were not pursued.

7.3.2 Rotorua possums : Observations at Massey University.

Possum R001 was very slow moving around the SAPU enclosure and died 36 hours after arrival. One week later a female possum (R002) was observed stumbling and wandering abnormally. This possum died later that evening or early the following morning. Two weeks after Rotorua possums were released into group accommodation, a third possum (R003) was observed in a depressed state and reluctant to move, appearing both incoordinated and lame in the left front leg. Possum R003 was killed humanely and fresh tissues collected. The fourth possum (R004) collected from Rotorua had a dependent joey and was moved to an adjacent cage where it remained free of signs of disease for two months. The two original resident possums did not show clinical signs of disease, were surplus to survey requirements, and were humanely destroyed.

Possum R005 was submitted five weeks after the first four possums and was released into the SAPU enclosure with six resident possums from the West coast of the South Island. Possum R005 was in very poor condition and had mild dermatitis associated with wet fur at the base of the tail. All possums frequently shared one nesting site. This possum appeared to fully recover and was killed humanely after one month in captivity. No possums in contact with R005 developed clinical signs of disease.

7.3.3 Rotorua possums : Necropsy and histopathology results.

R001 - This possum was judged to have been dead for less than 12 hours by the presence of rigor and minimal gas distension of bowel loops. The ambient temperature was low ($<12^{\circ}\text{C}$) and the carcase remained in shade until discovery. An open, granulating wound 2.5 cm in diameter was present at the base of the tail and a similar lesion was present on the dorsal aspect of the left metacarpus. Abdominal fat reserves were absent. The stomach contained ingesta, comprised of apple and the pelleted ration, and the small intestinal mucosa appeared reddened. The lungs contained areas of focal congestion and emphysema was noted in peripheral portions of the caudal lung lobes.

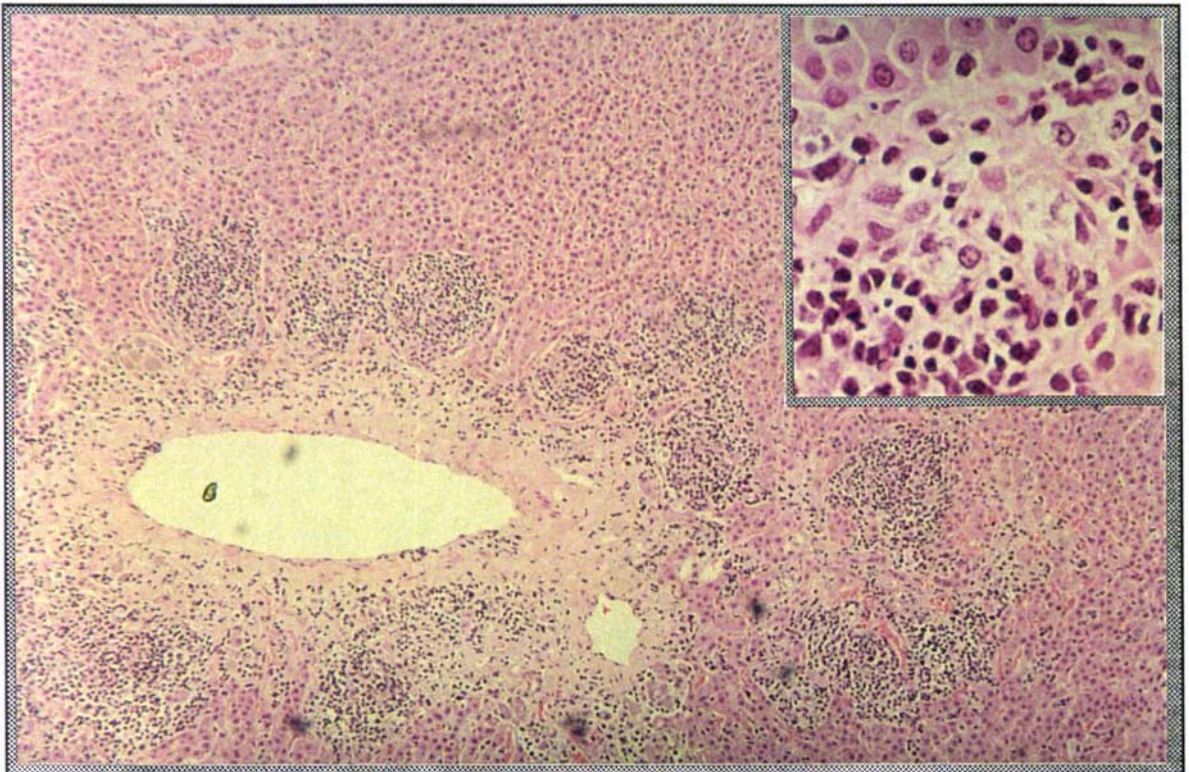
A mild, nonsuppurative meningo-encephalitis and choroiditis was present and lymphocytes and plasma cells predominated in cuffs around blood vessels (figure 7.1). Subcapsular and focal interstitial nephritis was observed as a chronic process with aggregations of lymphoid and plasma cells centred on, but not exclusive to, blood vessels. In the bladder, a chronic, mild inflammatory process was present. Foci of lymphocytes and plasma cells were both associated with and independent of blood vessels. Within the myocardium a few subacute inflammatory foci were identified consisting of lymphoid aggregations between fibres. Hepatic veins were surrounded by accumulations of mononuclear inflammatory cells, which were frequently focal and extended into the liver parenchyma (figure 7.2). Some inflammatory foci appeared independent of hepatic blood vessels. Histological evidence for pulmonary congestion and emphysema were present but not considered significant. A cause of death was not determined.

Figure 7.1 Section of brain from possum R001; H & E stain (x 430).



Nonsuppurative meningitis : Plasma cells and lymphocytes are the predominant cell types.

Figure 7.2 Section of liver from possum R001; H & E stain (x 85).



Nonsuppurative hepatitis : Mononuclear inflammatory cell foci are frequently adjacent to blood vessels. High power (x 430) insert of inflammatory focus : Remnants of degenerating and necrotic hepatocytes are visible. Many of the infiltrating mononuclear cells have pyknotic nuclei.

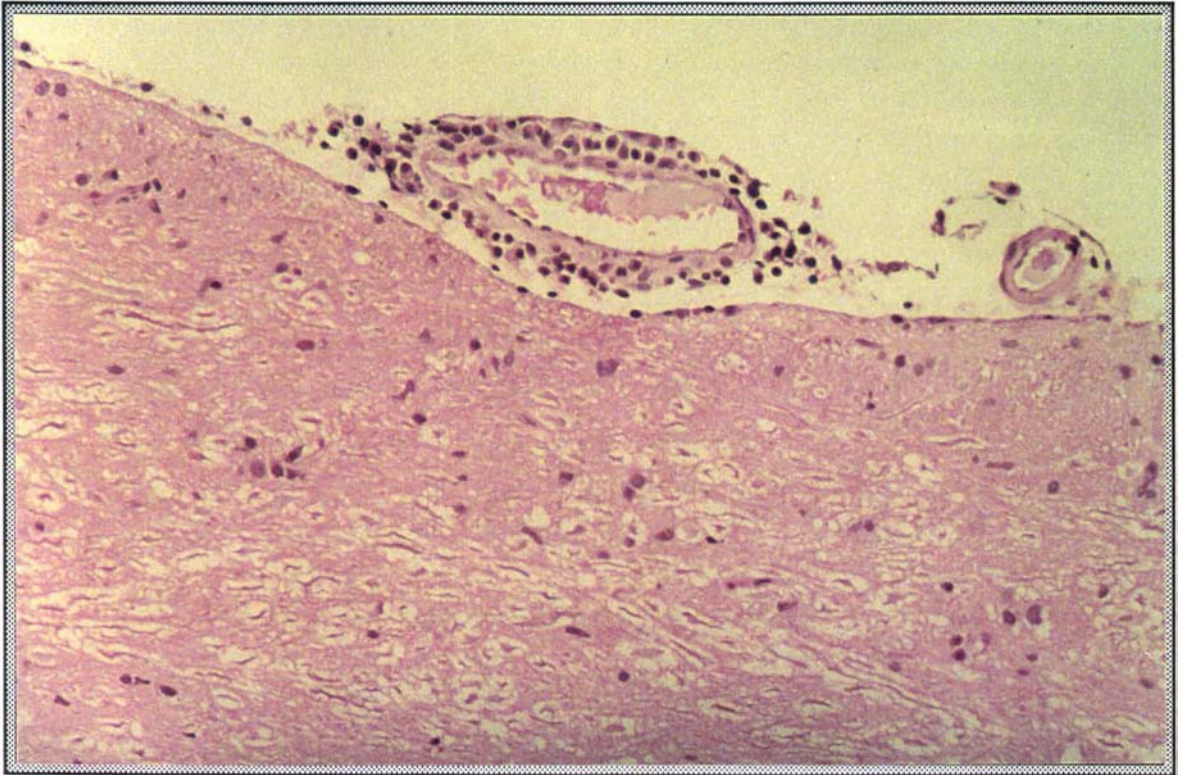
R002 - This possum was judged to have been dead for greater than 12 hours by the partial relaxation of rigor, distension of intestines with gas and the mottled appearance of the liver where there was contact with the stomach. R002 was in good overall condition and had generalised subcutaneous crepitus in the region of the ventral abdomen. The mucosa of the duodenum and jejunum was reddened, the liver was pale and the kidneys had multiple 2-3 mm darkly pigmented foci beneath the capsule.

Changes in the brain consisted of a mild, multi-focal, nonsuppurative encephalitis and choroiditis consisting of lymphocytes and plasma cells. There was a mild multifocal interstitial nephritis. Changes in the myocardium were as for possum R001. Perivascular infiltration of mononuclear inflammatory cells in the liver was relatively mild compared to possum R001. A cause of death was not determined.

R003 - The elbow and antebrachium of the left foreleg was swollen. A gelatinous exudate extended along oedematous tissue planes. Abdominal fat reserves were reduced. The lungs were pale with a "honey combed" emphysematous texture that was gritty when cut. There was a low grade dermatitis at the base of the tail.

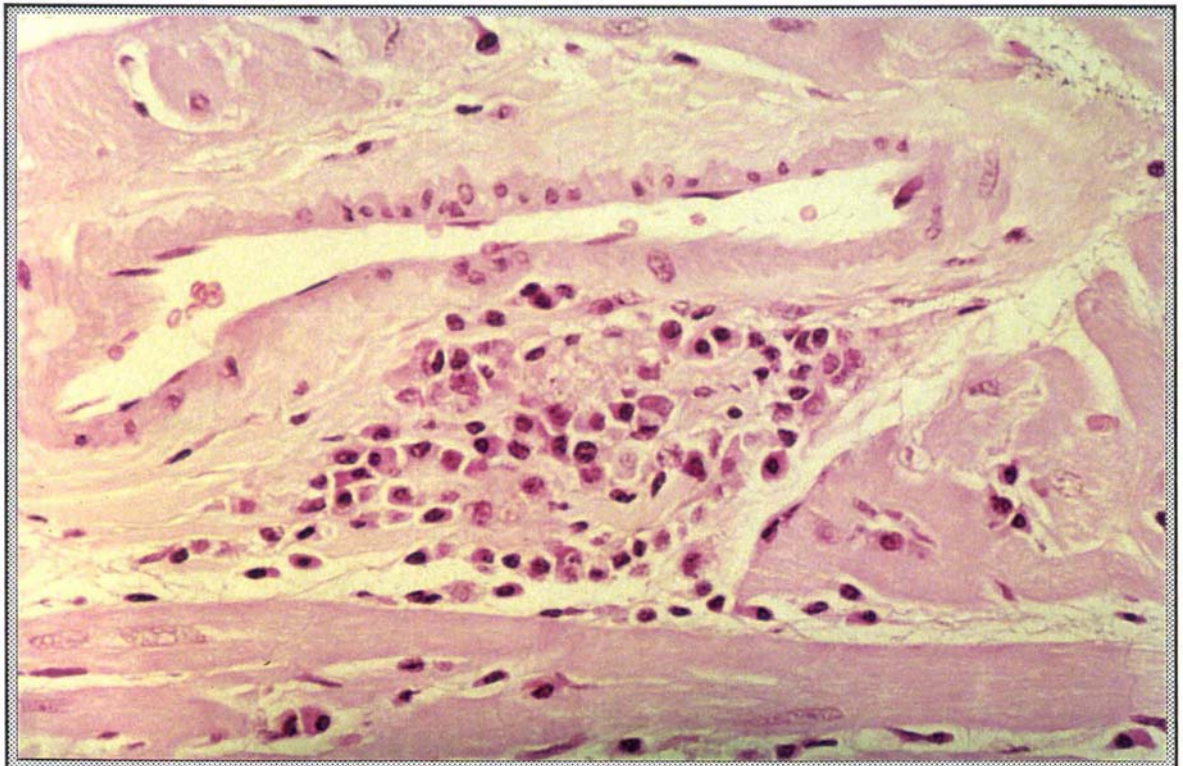
There was a mild, diffuse, nonsuppurative meningitis. In discrete foci, meningitis was moderate and inflammatory cells preferentially surrounded small venules (figure 7.3). Foci of mononuclear inflammation containing lymphocytes, plasma cells and macrophages were present in the myocardium, between muscle fibres, and in connective tissue locations adjacent to blood vessels (figure 7.4). Gram positive bacteria and a heavy infiltration of polymorph neutrophils and mononuclear inflammatory cells were associated with the elbow lesion. Chronic, widespread interstitial nephritis and cortico-medullary calcium deposition was present together with renal tubular cell swelling. Calcium deposition was also extensive in the lung and was demonstrated in alveolar septae by the Van Kossa stain. Protein and fibrin exudate was associated with necrotic cells, macrophages and giant cells in alveolar spaces. The cause of death was tentatively attributed to bacterial toxæmia superimposed on chronic severe inflammation (kidney and lung) and associated loss of mineral homeostasis (metastatic calcification).

Figure 7.3 Section of brain from possum R003 ; H & E stain (x 215).



Nonsuppurative meningitis : Plasma cells and lymphocytes are the predominant cell types and inflammatory cell accumulations demonstrate a venous bias.

Figure 7.4 Section of myocardium from possum R003 : H & E stain (x 430).



Nonsuppurative myocarditis : Mononuclear inflammatory cells infiltrate a small focus of cellular degeneration within myocardial connective tissue.

R005 - This possum was young and in light condition with a small amount of abdominal fat. The liver was pale and the bladder contained opaque, cloudy urine in which small crystals were detected.

A moderate to severe degree of fatty change and associated hepatocyte degeneration was present in the liver. Multiple foci of mononuclear inflammatory cells were identified in the renal cortical interstitium and a few calcium precipitates were detected in the medullary interstitium and in collecting tubules adjacent to the renal pelvis.

R006 - A large blood clot was present in the abdomen and a haematoma extended along the ventral aspect of the spinal cord. The left caudal lobe of the lung was distended and discoloured with dark blood. These lesions were presumed to have resulted from trauma.

The renal interstitium contained occasional foci of mononuclear inflammatory cells. A diffuse increase in mononuclear inflammatory cells was noted in liver sinusoids and these also occurred in small clusters. Hepatocytes and macrophages contained significant amounts of bile pigment. Mononuclear cells (lymphocytes, plasma cells and macrophages) and occasional polymorphonuclear cells were seen in inflammatory cuffs around larger hepatic vessels. The liver lesion was described as a mild, diffuse mononuclear leucocytosis. The brain and meninges were free of inflammatory foci. The lesions associated with death by traumatic misadventure were not relevant to investigation of an infectious disease process.

R007 - This possum was in average condition. No gross lesions were detected and there were no significant histological changes in the brain, kidney, liver or heart.

7.3.4 Rotorua possums : Tissue culture, cytology and haemadsorption.

R001 - Cells from prostate explants grew out vigorously to form a monolayer of healthy epithelial cells. This primary monolayer did not re-establish following attempted passage. Fibroblastic cells advanced to partial confluency from adrenal gland and lung tissue for two weeks before gradually deteriorating. Deterioration was observed as a prolonged stationary phase in which fibroblastic morphology became more pronounced prior to monolayer detachment. Cells from salivary gland tissue, tonsil, bladder and kidney grew out to varying confluency (15 - 60%) on varying time scales (8 - 19 days) to maximum coverage. Primary cells from these tissues had a fibroblastic form initially but were more epithelial at their peak of growth. Once stationary, epithelial cells from the above tissues became vacuolated and appeared to be 'stretched' to maintain coverage. This deterioration was gradual and prolonged. Passage of the above primary explant cultures (3.2.8, figure 3.2) did not detect viral CPE in multiplates or cytological changes in chamber slide monolayers. Haemadsorption assays were negative.

R003 - Partial monolayers were formed by outgrowth from prostate, salivary gland, kidney and lung tissue fragments. The prostate cell monolayer detached, was passaged and re-established to a limited extent. The pattern of deterioration of primary cells was the same as for possum R001 (described above). Subsequent explant primary culture passage (3.2.8) of kidney, lung, salivary gland and prostate cell outgrowths was negative for CPE, haemadsorption and abnormal chamber slide cytology.

R005 - Fibroblastic outgrowth was observed with kidney, salivary gland, bladder and tonsil tissue explants. Healthy radial outgrowths established early but deteriorated, as described above, before confluency in all tissues. Primary explant cultures were passaged as described above. No CPE, haemadsorption and abnormal chamber slide cytology was observed. Samples of trigeminal ganglia, dorsal root ganglia, brain stem, tonsil, heart, and buffy coat cells from possum R005, co-cultivated directly onto marsupial monolayers (3.2.5), were also negative for CPE, haemadsorption and abnormal chamber slide cytology.

7.3.5 Transmission trials : Clinical signs, necropsy and histopathology results.

(i) Experiment one :

Possum 623 showed a small decrease in appetite from day three pi. By day ten appetite was low and from day 14 pi movement was impaired. Ataxia was recorded on day 16 pi. The pattern of disease onset for possum 624 was identical. Possums 623 and 624 were killed 18 and 20 days following inoculation respectively.

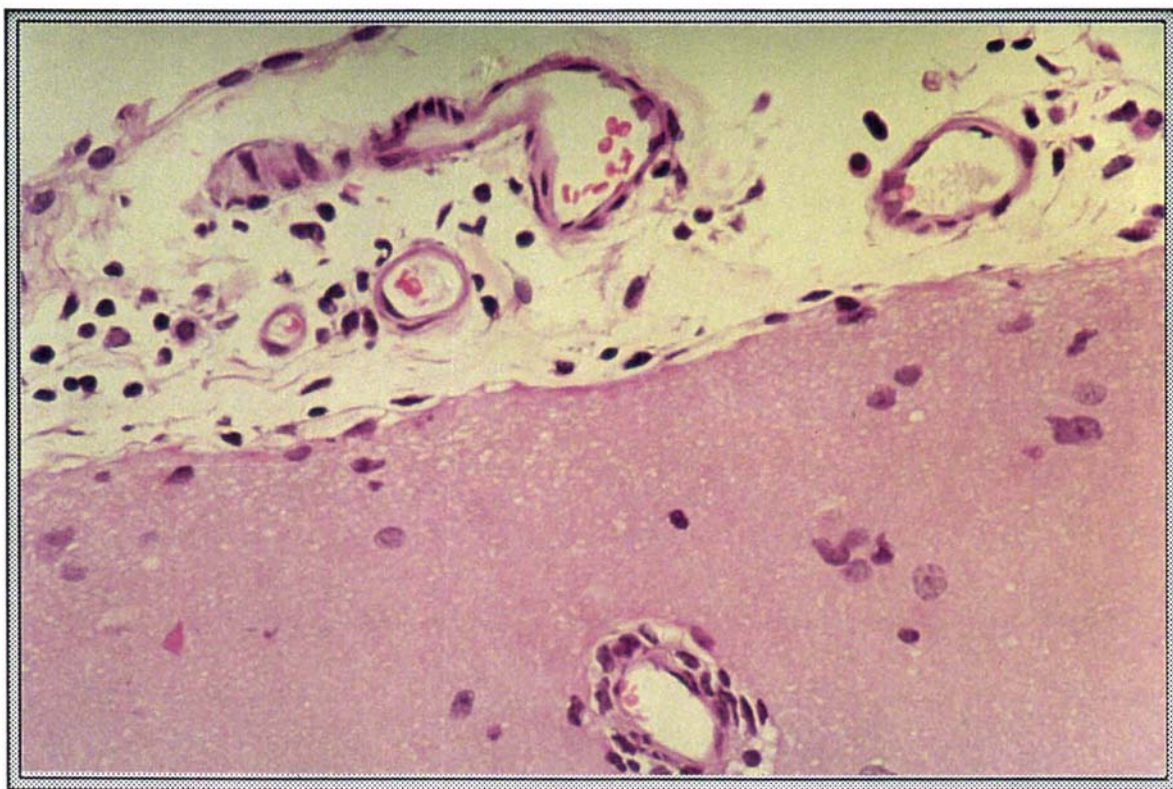
Abdominal fat reserves in possum 623 were minimal and the spleen appeared enlarged. This possum had a mild, focal nonsuppurative meningo-encephalitis. Perivascular cuffs of lymphocytes and plasma cells were identified in a few encephalitic foci but astrocytes also appeared to be relatively prominent in these lesions. The meninges were congested and there was evidence for a mild, focal nonsuppurative meningitis (figure 7.5). A venous bias in the distribution of mononuclear inflammatory cells was noted and mild focal choroiditis was observed in some tissue sections.

A moderately severe interstitial nephritis was present in which lymphocytes and plasma cells were predominant. The lesions were identified throughout the renal cortex and at the cortico-medullary junction where inflammatory cells were often associated with blood vessels. Sub-intimal infiltrations of these cell types were also identified.

The liver contained small focal parenchymal accumulations of lymphocytes and plasma cells. These inflammatory cells also surrounded small hepatic vessels and formed deep cuffs around central veins and portal vessels. The same inflammatory cell types were present in myocardial lesions between muscle fibre bundles, in areas of connective tissue and adjacent to the epicardium.

Possum 624 - No gross abnormalities were detected other than loss of fat reserves. Mild, focal nonsuppurative meningitis and choroiditis was present. Occasional foci of mild nonsuppurative encephalitis were detected. Lymphoid accumulations in the liver and associated with hepatic vessels were mild, with the exception of one significantly cuffed large vessel. Small areas of myocarditis (similar to possum 623) were observed and extensive interstitial nephritis, including lymphoid cell invasion adjacent to and beneath large blood vessel endothelia, was present.

Figure 7.5 Section of brain from possum 623, H & E stain (x 430).



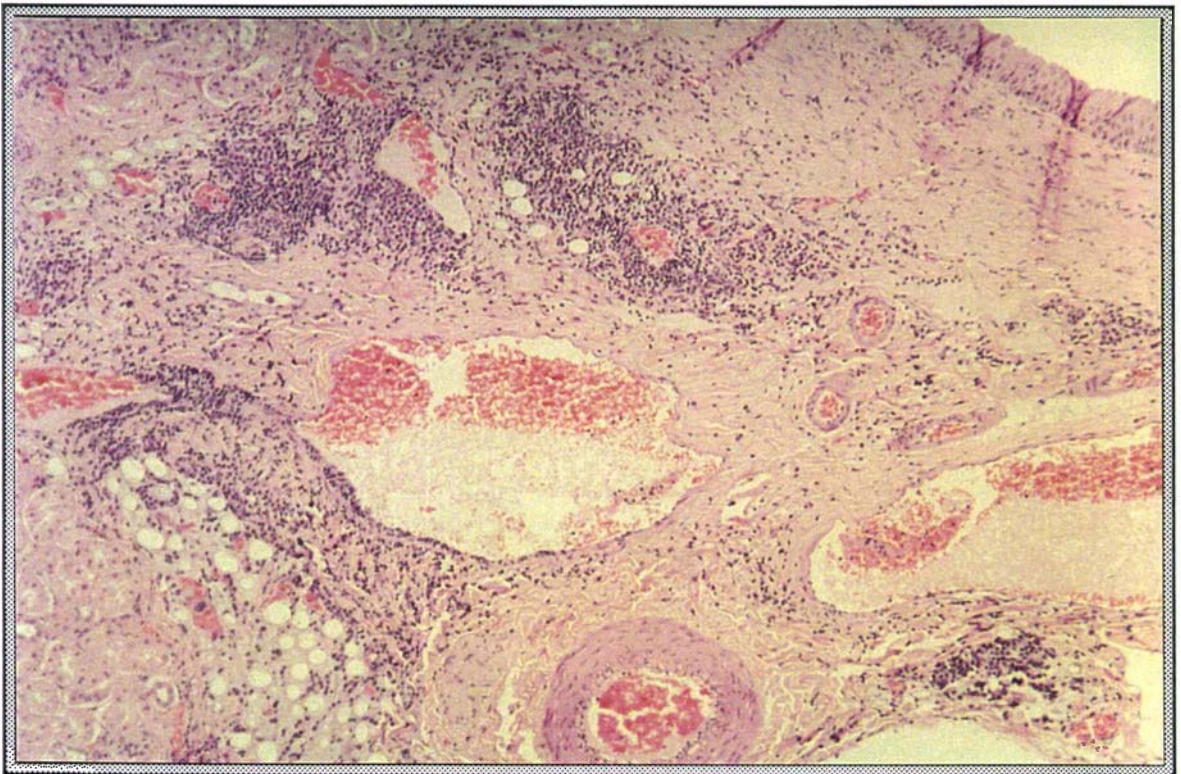
Nonsuppurative meningitis and adjacent encephalitis / vasculitis : Plasma cells and lymphocytes can be identified in focal areas of inflammation.

(ii) Experiment two :

Possum 638 became increasingly hyperactive and aggressive from day five pi and appeared to cough or choke on one occasion whilst drinking. From days 12-16 pi, possum 638 became progressively lethargic and appetite declined. From day 17 pi several abnormal movements, ranging from ataxia to involuntary shaking and head bobbing, were recorded. Possum 638 was considered to have severe neurological impairment and was killed 22 days pi. Abdominal fat reserves were almost absent. Mild non-suppurative meningitis was observed and plasma cells were present in astrocytic cuffs in the brain. A localised focus of inflammatory cells, including plasma cells, was identified in one spinal cord section. In the liver, mononuclear inflammatory cells were present in focal parenchymal locations and in prominent peri-vascular cuffs around large hepatic vessels. Interstitial nephritis, perivascular and sub-intimal inflammation associated with plasma cells and lymphocytes was similar to kidney lesions seen in possum 623. A mild myocarditis, including mononuclear inflammatory cell accumulations beneath blood vessel endothelia, was present.

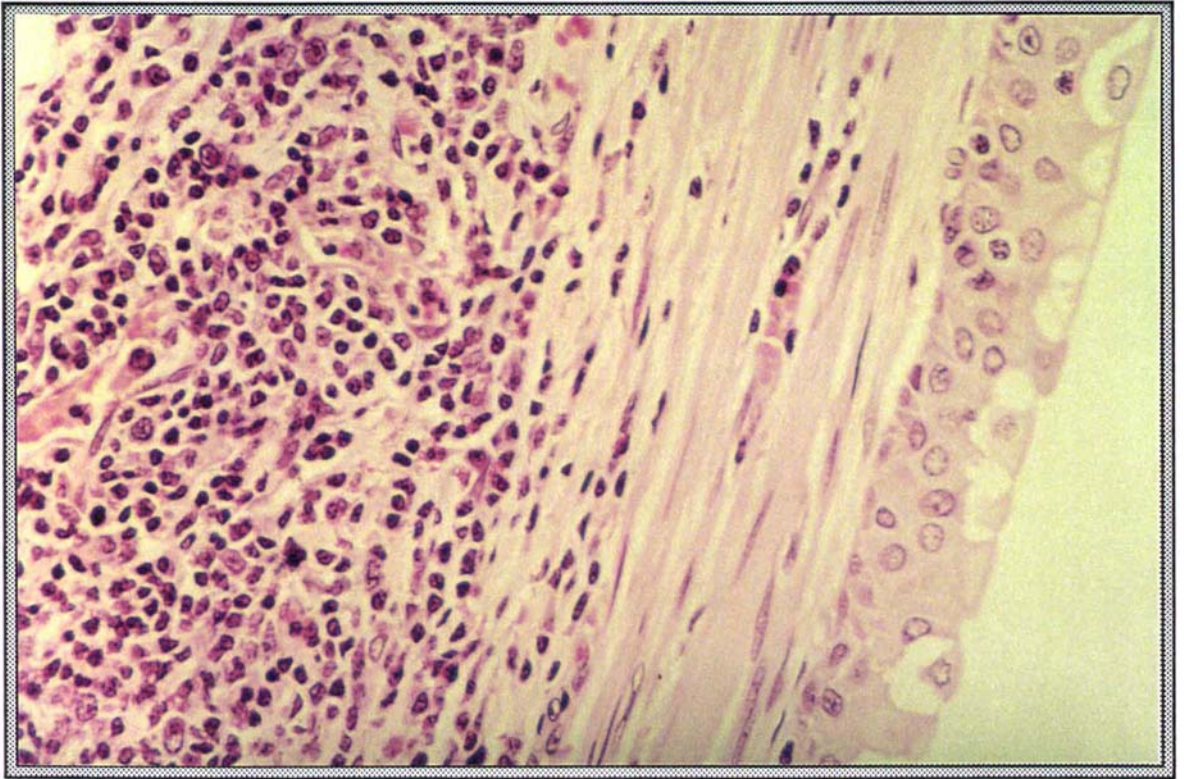
Possum 639 remained co-ordinated and active for the duration of the trial (26 days) but was judged to be more active during the second fortnight in captivity than in the two week period immediately following inoculation. At necropsy, fat reserves were reduced. A localised lesion, consisting of lymphoid mononuclear cell aggregation at the junction of white matter and grey matter in the spinal cord, was identified in the CNS. Prominent cuffs of plasma cells and lymphocytes were present in the liver and increased sinusoid cellularity included these cell types and nucleated RBCs. A moderate, multifocal, interstitial nephritis with sub-intimal and peri-vascular accumulations of plasma cells and lymphocytes was observed. In possum 639 these lesions appeared more severe in the renal pelvis (figure 7.6 and figure 7.7).

Figure 7.6 Section of kidney from possum 639 : H & E stain (x 86).



Dense, focal accumulations of mononuclear inflammatory cells adjacent to the renal pelvis and frequently associated with blood vessels.

Figure 7.7 Section of kidney from possum 639 : H & E stain (x 430).



Focus of mononuclear inflammatory cells adjacent to the transitional epithelium of the renal pelvis.

(iii) Experiment three :

Possum 633 was difficult to handle (aggressive and defensive) during the early post-inoculation period. Appetite was low for the first fortnight pi but progressively increased. From day 12 to day 23 pi possum 633 was slow and apathetic but was never observed to be obviously ataxic. This possum was co-ordinated and appeared more alert at it's final assessment prior to euthanasia (26 days pi) than during earlier assessments.

Abdominal fat reserves in possum 633 were reduced. Occasional plasma cells were seen in the mildly congested meninges. No other lesions in the CNS were identified. Mononuclear inflammatory cells were observed in parenchymal foci in the liver and together with nucleated RBCs in sinusoids. Mononuclear cell cuffs around large hepatic blood vessels were moderately extensive but did not closely border the vascular channel as did cuffs in possum 623. Extensive subcapsular and cortical interstitial nephritis was present. Eosinophilic, proteinaceous material distended vessels and collecting tubules and there were substantial areas of peri-vascular inflammation adjacent to the renal pelvis, including sub-intimal vascular accumulations of lymphocytes and plasma cells. Small foci of lymphocytes and plasma cells were detected beneath the epicardium and within the myocardium.

Possum 634 showed an early decrease in appetite and some uncertainty of movement at eight days pi. From day eight pi possum 634 appeared depressed, showing little interest in the handler and on day 12 was unsteady, quiet and slow. Possum 634 was difficult to arouse and lethargic for the duration of the trial and, although the wet food ration was always eaten overnight, died 21 days pi.

Abdominal fat reserves were absent and abdominal organs were partially discoloured where there was contact with distended bowel loops. There were no lesions observed in the CNS or other tissues examined. The liver, kidney and heart contained circumscribed colonies of bacteria within capillaries and macrophages. No inflammatory response and no significant accumulations of mononuclear cells were associated with these colonies.

Possum 635 - Served as a non-inoculated control for the four possums in experiments two and three. This possum remained alert and co-ordinated throughout the trial and was killed 27 days pi. No clinical signs were observed and no gross abnormalities were detected at necropsy. A diffuse, mild, interstitial nephritis was observed but no abnormalities were detected in other tissues.

Cultivation and extended passage of peripheral blood and WBC enriched inocula, from possums 633 and 634, as part of experiment three did not demonstrate any CPE in PCEF monolayers.

(iv) Experiment four :

Possum 675 showed an early decrease in appetite. Ataxia was suspected at day 13 pi and by day 17 this possum was slow and inco-ordinated. Ataxia and depression were marked for the remainder of the trial and possum 675 was humanely killed 23 days pi.

Abdominal fat reserves were absent and skeletal muscle mass appeared diminished. Mild, focal, nonsuppurative meningitis and mild, interstitial nephritis was present. Protein was present in distended proximal convoluted tubules and mineral deposits were identified in tubules and interstitial locations. Perivascular cuffs in the liver appeared to contain

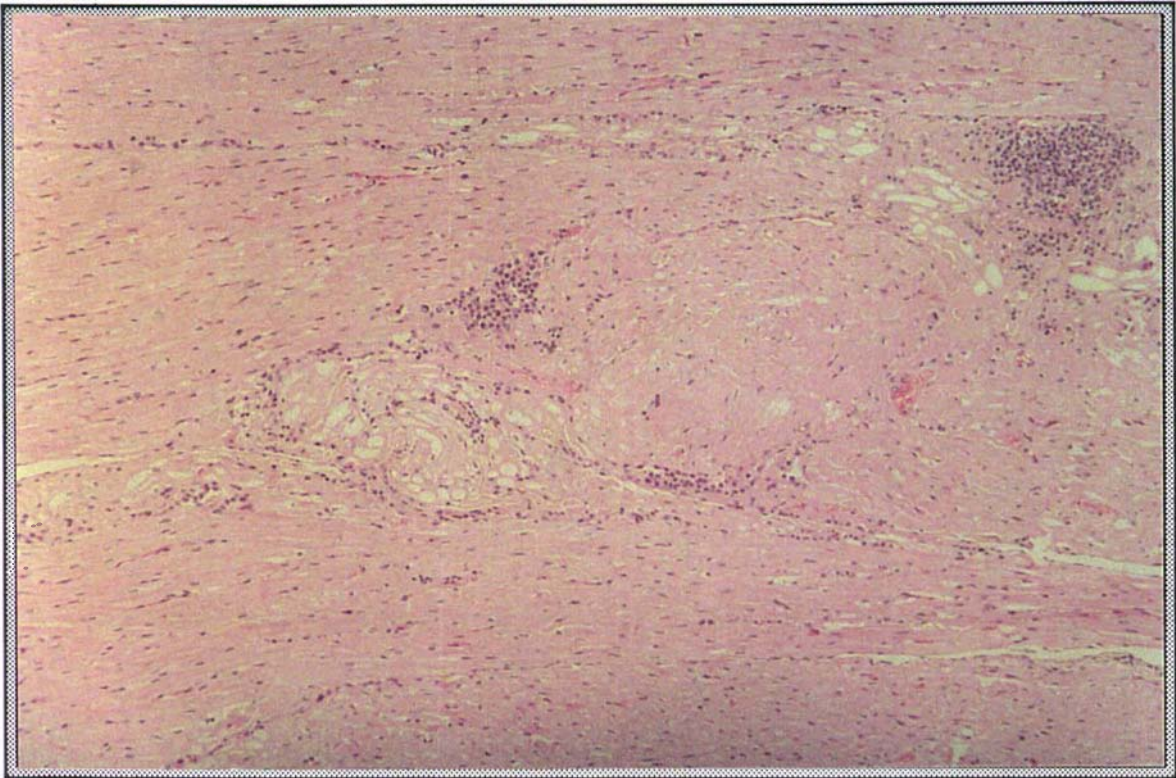
degenerating hepatocytes or macrophages in addition to the usual lymphocytes and plasma cells seen in possum 638 and others. In the bladder, an increase in the number of mononuclear inflammatory cells was observed.

Possum 677 was slower to develop neurological signs. Investigative behaviour and alertness was reduced from day 17 pi and handling became progressively easier. Obvious ataxia was detected from day 22 pi. Opisthotonus was recorded on day 25 pi and possum 677 was killed 36 days pi.

Some abdominal fat reserves remained and the liver appeared fatty. The residual urine volume contained a gritty sediment. Mild, multi-focal nonsuppurative meningitis was observed in the brain and spinal cord. Mononuclear inflammatory cells were present in the liver parenchyma and in deep cuffs around hepatic vessels. Multifocal interstitial nephritis had a slight cortical bias. Association of mononuclear inflammatory cells with renal vasculature, including sub-intimal foci, had a cortico-medullary bias. Mild, multifocal lymphoid accumulations were present in the bladder wall. Moderately large foci of lymphocytes and plasma cells were seen beneath the endocardium, between muscle bundles and in the loose connective tissue of the myocardium, often adjacent to blood vessels (figure 7.8 and figure 7.9).

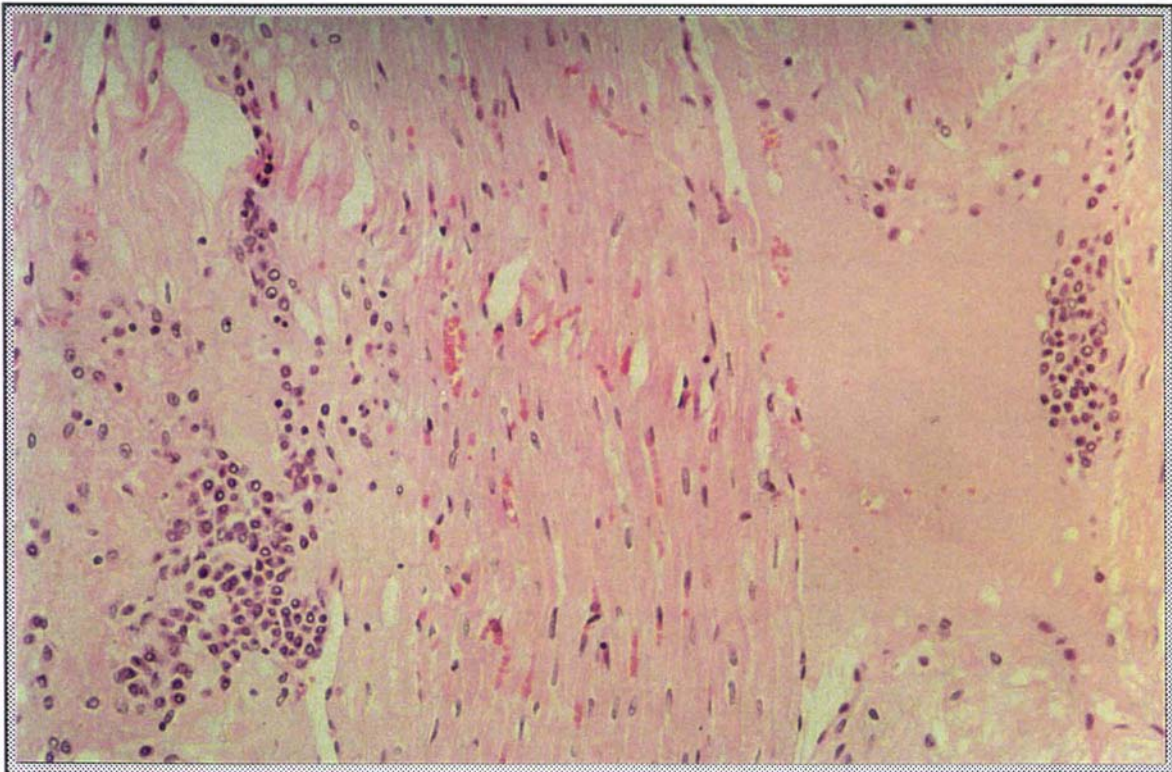
Possum 678 (control) gained weight and remained active and alert until it was killed, 36 days following inoculation with a non-infectious tissue suspension (6.2.2). Excess abdominal fat and a pale fatty liver was noted grossly. Histologically, hepatocytes were distended with fat and many appeared to be degenerating. No other abnormalities were observed.

Figure 7.8 Section of myocardium from possum 677; H & E stain (x 86).



Myocarditis : Small, focal, mononuclear cell infiltrations are seen within the myocardium. Larger accumulations of these cells were also identified adjacent to the endocardium.

Figure 7.9 Section of myocardium from possum 677 : H & E stain (x 215).



Mononuclear inflammatory cells (predominantly lymphocytes and plasma cells) were identified between myocytes and were associated with blood vessels and loose connective tissue.

7.3.6 Comparison of the Rotorua syndrome with WPD : Clinical features.

Clinical similarities between the Rotorua syndrome and WPD were very strong as indicated below (Table 7.2). The two sample populations were not significantly different from each other for either 'onset of behaviour changes' or 'onset of ataxia' (P values of 0.5330 and 0.9455 respectively) for the possums which developed neurological disease. However, three possums challenged (IP) with tissue suspensions, from confirmed cases of the Rotorua syndrome, did not develop typical ataxia. Possum 634 was extremely depressed and lethargic but could walk slowly when encouraged. Possums 633 and 639 (to a lesser extent) appeared to be mildly lethargic and less mobile in the first fortnight following inoculation but were alert and active immediately prior to euthanasia. In contrast, possums inoculated with similarly prepared tissue suspensions from confirmed cases of WPD invariably developed severe neurological signs.

Possums inoculated with tissue preparations from possums with either WPD or the Rotorua syndrome lost similar amounts of body weight and condition during trial periods. Gross findings at necropsy in both groups of possums were very similar and consisted of loss of body condition and depletion of abdominal fat reserves (4.3.5).

Table 7.2 Onset of clinical signs of WPD and Rotorua syndrome.

DISEASE ENTITY	MEAN DAYS TO BEHAVIOUR CHANGE	MEAN DAYS TO ATAXIA
WPD	10.1 (n = 33)* range 5 - 20	16.5 (n = 38)* range 11 - 25
Rotorua disease	11.1 (n=7) range 7 - 17	16.0 (n=6) range 12 - 22

*** Possums exposed to WPD agent by natural contact not included.**

7.3.7 Application of the WPD case definition to the Rotorua syndrome.

Possums from Rotorua and possums challenged with tissues derived from R001 were numerically scored for WPD (Table 7.3). Of the wild caught possums numerically assessed, only R001, R002 and R003 (each with a score of nine) met the criteria for classification as WPD. Possums R005, R006 and R007 (scoring two, three and one) had few or no histological changes consistent with WPD.

Of the transmission study possums, 623 and 624, given R001 spleen and liver inocula, were positive, each with a score of 10. Possum 638, inoculated with brain and spleen from possum R001, obtained a positive score of 11 but possum 639, also inoculated with R001 tissues (liver and spleen), gained a score of three which was below the diagnostic cut off. Possum 633, inoculated with a liver, spleen and brain preparation from possum 624, developed a mild form of the clinical disease with sufficient histological evidence for a numerical score of seven. Possum 634, inoculated with a filtered liver, spleen and brain suspension from possum 623, did not develop histological lesions typical of WPD but obtained a score of four, for non-specific behavioural changes and weight loss. Possums 675 and 677, inoculated with pooled liver, spleen and brain suspensions from possum 638, developed WPD-like clinical signs and lesions with scores of 10 and 9 respectively. Six out of eight inoculated possums met the numerical criteria for a diagnosis of WPD but only five of these eight were unambiguously ataxic and had lesions in the CNS. Morbidity for the experimentally transmitted disease was 75%. Control possums 635 and 678 had numerical scores of 1 and 0 respectively.

Table 7.3 Classification of Rotorua syndrome possums using WPD criteria.

Possum Number	Inoculum + Route	CLINICAL SIGNS					HISTOLOGICAL SIGNS				WPD Score
		Ataxia	Immobile	Behaviour	App. ↓	20% Wt. ↓	CNS	Liver	Kidney	Heart	
R001	field case	+++	-	+	nr	nr	++	+	+	+	9
R002*	field case	+++	-	+	nr	nr	++	+	+	+	9
R003*	field case	+++	-	+	nr	nr	++	+	+	+	9
R005**	field case	-	-	+	nr	nr	-	-	+	-	2
R006**	field case	nr	nr	+	nr	nr	-	+	+	nr	3
R007**	field case	nr	nr	+	nr	nr	-	-	-	-	1
623	R001 ip	+++	-	+	+	nr	++	+	+	+	10
624	R001 ip	+++	-	+	+	nr	++	+	+	+	10
633	624 ip	-	+	+	+	+ 32% ↓	-	+	+	+	7
634	623 ip	-	+	+	+	+ 20% ↓	-	-	-	-	4
635	control	-	-	-	-	- 1% ↑	-	-	+	-	1
638	R001 ip	+++	-	+	+	+ 26% ↓	++	+	+	+	11
639	R001 ip	-	-	-	-	+ 22% ↓	-	+	+	nr	3
675	638 ip	+++	-	+	+	+ 32% ↓	++	+	+	-	10
677	638 ip	+++	-	+	-	- 6% ↑	++	+	+	+	9
678	control	-	-	-	-	- 31% ↑	-	-	-	-	0

Possums affected with a disease syndrome very similar or identical to WPD were scored using the numerical criteria developed for WPD in chapter four. Captured possums * from Rotorua developed the disease by contact with possum R001 (original Rotorua possum with neurological disease). Experimental possums developed the disease following intra-peritoneal (ip) inoculation with tissues homogenates derived from possum R001. Further possible natural cases ** were investigated. + / ++ / +++ = Presence of a clinical sign or histological lesion associated with WPD. Changes indicating CNS involvement are weighted (4.2.6).

App ↓ = Appetite decrease. 20% Wt. ↓ = Loss of 20% of body weight during the course of captivity / experiment. nr = not recorded.

7.4 DISCUSSION

Mononuclear inflammatory cell foci in the CNS and myocardium were detected in wild caught possums submitted and held for investigation of an infectious disease process. These lesions were not a feature of previously recognised disease, or an incidental finding, in possums and a viral aetiology was suggested (Michèle Cooke, pers. comm.). The death in captivity of two apparently healthy possums from Rotorua was also an indication for an infectious disease even though necropsy and histological findings were inconsistent and no further illness was seen in possums sharing the single enclosure and one main nesting site.

To investigate a viral aetiology, fresh tissues from a number of potentially affected possums were subjected to the standard and extended tissue culture survey protocols (chapter 3). Co-cultivation, haemadsorption and chamber slide examination procedures were negative. Deterioration of primary cell cultures was considered to be due to poor primary cell survival following explantation (7.3.4) and no CPE could be detected in subsequent co-cultivation assays. When no cytopathic agent could be recovered, and following the description of a similar syndrome in captive possums, transmission studies which confirmed an infectious aetiology were undertaken. During transmission studies, and after similarities between WPD and the Rotorua syndrome had been noted, blood was collected before clinical signs of the WPD-like illness. This was done within the proposed incubation period and (potentially) before a specific immune response in an attempt to cultivate infectious virus. No virus was isolated and this result was considered together with negative results from a previous series of experiments to isolate WPD virus (5.2.2) and is discussed in chapter five.

The ataxia and wasting which developed in typically affected possums inoculated with infectious Rotorua syndrome tissues was identical to the clinical signs of WPD. However, clinical signs (behavioural changes) in possums inoculated with the Rotorua syndrome were more variable than the clinical signs associated with WPD. In contrast with WPD, two possums inoculated with the Rotorua syndrome did not develop typical neurological signs.

Histological lesions in possums affected with the Rotorua syndrome were very similar to changes seen in possums with WPD. The cell types in the inflammatory cell accumulations were almost always lymphocytes and plasma cells. Rotorua possums, in which nonsuppurative meningo-encephalitis was well developed, had lesions identical in character and distribution to the CNS lesions seen in WPD possums. However, Rotorua possums usually had a mild, nonsuppurative, meningitis whereas possums with WPD more often had a moderate, nonsuppurative meningo-encephalitis. The histological lesions seen in the liver and kidney of Rotorua possums were considered to be almost identical to the lesions seen in these tissues from possums with WPD. In Rotorua possums, perivascular mononuclear cell accumulations in the liver varied from mild to extensive. In extensive cases, the majority of vessels were affected, cuffs involved the entire perimeter of the vessel and inflammatory cells also occupied intra-parenchymal locations. In the kidney the number and size of interstitial inflammatory cell foci also varied. Inflammatory cell infiltrations were not always seen in the sub-intima or surrounding the vessels in the kidney.

Differences between WPD possums and Rotorua syndrome possums were limited to the extent and severity of the histological lesions. In spite of variation within the respective WPD and Rotorua syndrome possums, lesions in the Rotorua possums were considered to be less severe. In particular, two possums inoculated with the Rotorua syndrome neither displayed obvious ataxia nor developed observable lesions in the CNS. They did, however, lose weight and demonstrate typical lesions in other tissues. The Rotorua possums presented a greater number of lesions considered to be secondary or unrelated to the underlying infectious agent (for example, metastatic calcification).

Overall, the similarities between the two disease entities are striking and it is hypothesised that the Rotorua syndrome is a variant of WPD. The terms 'Invermay strain' (WPD^{IS}) and 'Rotorua variant' (WPD^{RV}) were adopted. Thus, a WPD-like syndrome was described in the North Island and in a wild possum population for the first time.

Three differences between the two syndromes are considered significant.

1. Possums inoculated with infectious WPD^{RV} tissue suspensions did not invariably develop neurological disease or lesions in the CNS. Although tissue suspensions from Rotorua syndrome possums varied in composition, possums challenged with similarly concentrated WPD^{IS} derived tissues or the standard inoculum always contracted WPD following intra-peritoneal inoculation.
2. Even when there was obvious ataxia in possums inoculated with WPD^{RV}, the histological lesions in the CNS were milder than equivalent changes detected in the CNS of possums inoculated with WPD^{IS}.
3. Possums inoculated with WPD^{RV} that did not develop CNS signs or lesions had evidence of WPD^{IS}-like histological changes in extra-neural tissues.

The study of WPD^{RV} has supported the suggestion that WPD is not primarily a neurological disease (4.5). A severe disease characterised by weight loss and mononuclear cell inflammation in a variety of organs is consistent with this hypothesis in the absence of CNS signs or lesions in some possums and mild CNS signs in others. This is demonstrated in the inoculated possums which had obvious characteristic lesions in several organs, including the liver and kidney, yet had no, or minimal, lesions in the CNS. Only one possum with WPD^{RV} had extensive CNS lesions whereas seven out of 11 were considered to have significant, as opposed to mild, liver lesions. Lesions in the kidney were consistently comparable with the range (severity and distribution) of lesions seen in WPD^{IS} with only one case described as mild. One inoculated possum (639) that did not develop detectable neurological disease had relatively severe lesions in the kidney (figures 7.6 and 7.7). Two possums submitted to RAHL, for investigation of an infectious disease, were observed to have WPD-like histological changes in extra neural locations alone (7.3.1) and a third possum had a mild nonsuppurative meningitis and WPD-like histological changes in other tissues (Fairley, pers. comm.).

There are many precedents for variable neuro-tropism amongst viruses that affect the nervous system. One example, Newcastle disease virus of poultry, has strains of varying virulence and strains of differing tropism (Kouwenhoven, 1993). Therefore, WPD^{IS} and WPD^{RV} may be different strains of the same virus.

One alternative hypothesis is that the two diseases are identical and that the duration of the transmission experiments involving WPD^{RV} was insufficient for expression of the full virulence of WPD^{RV}. Limited support for this idea comes from naturally infected possum R001 which had a florid, nonsuppurative meningo-encephalitis typical of the CNS lesions seen in WPD^{IS} possums. Possum R001 was affected with WPD^{RV} for an unknown length of time allowing speculation that the lesions in the CNS associated with WPD^{RV} may require a longer incubation period. However, clinical correlates of the two proposed strains of WPD are very similar (7.3.6) and several possums inoculated with WPD^{RV} and killed when severely ill, including one kept for 36 days pi, did not have enhanced lesions in the CNS. With the exception of mild lesions in the CNS, other indications for a strain difference came from possums inoculated in the second and third transmission trials. Therefore, a second alternative to be considered is that infectious tissue preparations inoculated into these possums contained a lower concentration of the infectious agent or that the preparation protocols altered some aspect of the virulence of WPD^{RV}.

In order to determine the relationship between WPD^{RV} and WPD^{IS} these two agents need to be further characterised. Classification of WPD^{IS} and WPD-like agents with recognised RNA viruses is likely to be aided by genome comparisons as morphological and physicochemical studies have failed to definitively classify the agent to date (chapter 5, O'Keefe, 1996; O'Keefe *et al.*, 1997; O'Keefe and Wickstrom, 1998a). Sequence information from WPD^{IS}, once obtained, will enable the development of PCR and nucleic acid hybridisation techniques for comparison with WPD^{RV}.

Further *in vivo* challenge experiments to investigate clinical and histological variations seen with WPD^{RV} infection may determine whether reduced pathogenicity (and/or neurological tropism), compared to WPD^{IS}, is real or an artefact of experimental procedures. Ideally, a standardised infectious tissue suspension from WPD^{RV} possums should be prepared and titrated for meaningful comparisons with WPD transmission data. Serial *in vivo* passages may allow more fulminant CNS histopathology to be demonstrated or the observed differences to be upheld.

Following the description of WPD, several members of the N.Z. rural community volunteered anecdotal information indicating that ataxic possums had been identified in

various locations over the last twenty years. Sublethal poisoning or plant intoxication (marijuana) has often been used by members of the public to explain these observations and no investigations have previously sought to identify an infectious disease. Toxoplasmosis is a recognised differential diagnosis for CNS disease of possums (Presidente, 1984; Canfield, 1990; Hartley, 1993) and while it causes epidemic abortion in sheep in N.Z. (Blood *et al.*, 1983a), toxoplasmosis has only been diagnosed sporadically in possums. Histological evidence supporting the occurrence of WPD in several localities in New Zealand has been collated by the Ministry of Agriculture and Fisheries (anon. 1995; anon. 1995a; anon. 1997; anon. 1998). The transmission studies reported in this chapter provide evidence that these lesions in wild possums, at least from the Rotorua district, are in fact associated with an infectious disease. Therefore, a confirmed WPD-like illness in the Rotorua district, histological evidence for WPD-like syndromes elsewhere and a considerable geographical distance between Invermay and the North Island foci of WPD-like syndromes suggests that WPD is more likely to be a newly recognised disease than a newly emergent disease.

It is unknown whether or not WPD-like disease(s) are more widespread in N.Z. Wobbly possum disease has not been diagnosed in Australia but specific investigation of archival material for cases where the histology is suggestive of WPD has not been reported. A disease entity characterised by severe encephalitis and retinitis has been described in New South Wales (Hartley, 1993) but it has neither been demonstrated to have an infectious aetiology nor suggested to be related to WPD.

Several research questions await investigation. Was WPD virus (or WPD-like viruses) imported with the possum from Australia or has the virus evolved from an unknown source to take advantage of an ecological opportunity that is only available in New Zealand? Has WPD (or WPD-like syndromes) been cycling in N.Z. causing undetected systemic illness for some time? Have strains of a well adapted possum virus increased in virulence to a point where the disease they cause is clinically apparent or has a constant level of WPD in a wild population been recognised due to greater research interest in this species? In order to answer some of these questions a serological test to screen possums for antibodies to WPD is required. When or if it is demonstrated that both WPD^{IS} and

WPD^{RV} are detectable in a single serological assay, the prevalence and distribution of these, and related agents, in the N.Z. and Australian possum populations may be investigated. A serological assay would also enable the scoring system and the hypothesis stating that WPD^{RV} is not primarily a neurological disease, to be tested. If the mildly affected or behaviourally normal inoculated possums, that developed WPD-like histological changes in tissues other than the CNS, were shown to have sero-converted following challenge with WPD^{RV}, then this hypothesis would be supported. As WPD or a close relative may be widespread in New Zealand, previous comments concerning the future direction of work on WPD^{IS} also apply to WPD^{RV} (4.4, 5.4).

Astute observations by members of the public have previously alerted investigators to the presence of new viral diseases. For example, feline immuno-deficiency virus was first recognised in a large, multi-cat household that was negative for feline leukaemia virus by the persistence of the owner who claimed her cats had an 'aids-like' illness (Pederson, 1991). The owner of the property where the Rotorua disease was first recognised deserves credit both for his observations and the time and effort he invested to bring WPD^{RV} to the attention of the scientific community. Wobbly possum disease was first described in a research establishment following the outbreak of an illness presenting with neurological signs. A WPD-like disease was first recognised as the Rotorua syndrome and the reporter's convictions for an infectious aetiology were upheld following the investigations described above. Local knowledge was able to rule out pesticides and plant intoxications were considered unlikely. First hand experience of normal possum behaviour for this locality suggested that an infectious agent was a genuine possibility when significant behaviour changes were identified. It has been previously noted that rabies virus infection in wildlife leads to a heightened visibility of animals with a neurological disease. Rabid animals (carnivores in particular) are more likely to be observed by the public due to changes to normal behaviour patterns. Aimless wandering and approachability during daylight hours were the features of the Rotorua disease that appeared most unusual and led to the recognition of WPD^{RV}. Diseased possums without CNS involvement may have retained a greater repertoire of normal (nocturnal) behaviour patterns and thus remain undetected.

7.5 SUMMARY

A disease syndrome in possums from the Rotorua district, characterised by unusual behaviour, was notified by a member of the public. Unexpected deaths in a group of captive possums from Rotorua indicated the possibility of an infectious aetiology and the histopathology was suggestive of a viral disease. Although no viral agents were isolated in tissue culture, an infectious aetiology was confirmed when a characteristic syndrome was reproduced following the inoculation of tissues from affected possums into naïve recipients. The clinical and pathological signs were very similar and frequently indistinguishable from WPD. It is hypothesised that this syndrome is caused by a variant of the WPD virus.

The major finding from preliminary investigations into WPD^{RV} is confirmation of a second focus of WPD or a WPD-like illness in the North Island of N.Z. The neurological syndrome recognised in Rotorua was determined to have a lower morbidity and appeared to be less neurotropic than WPD^{IS}. Two possums inoculated with infectious WPD^{RV} tissues were observed to have WPD-like lesions in extra-neural tissues when evidence for neurological involvement was minimal or absent. These findings suggest that WPD^{RV} is less pathogenic than the Invermay strain and also support the hypothesis that WPD is not primarily a neurological disease. However, it was not possible to exclude the possibility that minor differences in experimental protocols had contributed to an apparent difference between the disease syndromes associated with WPD^{IS} and WPD^{RV}.

Evidence for a WPD-like illness in a natural environment and distant from where the disease was first described suggests that WPD is not a newly emergent disease. Wobbly possum disease is probably a newly recognised disease uncovered as a result of increased research activity and observation of possums.

Chapter 8

PAPILLOMAVIRUS IN POSSUMS

8.1 INTRODUCTION

The relatively high profile given to the identification of viruses in possums resulted in submission of wart-like lesions from a field caught possum. Papillomas were identified on the tail of a possum that was captured repeatedly as part of a longitudinal study to identify patterns of spread of bovine tuberculosis between cattle, possums and other wildlife species (Pfeiffer and Morris, 1991). Possum D2105 was captured 56 times between November 1993 and September 1994. It was handled 13 times and papillomas were first noted in March 1994. At the end of the study the whole tail was submitted for investigation. No similar lesions were seen in 740 tagged possums over the five year duration of the study. A total of 239 possums from the survey area which were killed at the end of the study and 135 possums tagged since, have not revealed any other papillomatous lesions (Lugton, pers. comm.).

Presumed possum papillomas and a proliferative 'wart-like' lesion on the tail of this possum were investigated and the gross and histological features of the possum papillomas, including associated papillomavirus (PV) particles, described. Preliminary molecular biological investigations (Meekin; O'Keefe, data not presented) suggested a new PV type and further molecular characterisation provided sequence data for comparison with similar PV genomes and phylogenetic analysis. These investigations and attempts to transmit and propagate the virus *in vivo* are described in this chapter. A brief review of papillomaviruses has been presented (chapter 2) in order that hypotheses regarding the likely source of the possum papillomavirus could be developed. The potential use of this virus as a disseminating vector for biological control of possums is discussed briefly.

8.2 MATERIALS AND METHODS

8.2.1 Sample collection, handling and storage.

Shallow biopsies of the proliferative lesion were obtained under sedation with ketamine (40 mg/kg) during routine handling on two occasions (April and May 1994). Tissue samples for analysis by transmission electron microscopy (EM) were fixed immediately. Tissues were also fixed in neutral buffered formalin (10%) and prepared for routine histological examination (haematoxylin and eosin). Remaining tissues were prepared for virus particle identification (negative contrast EM) and stored at -70 °C.

8.2.2 Electron Microscopy (EM).

(i) Preparation of papilloma for transmission EM : All reagents and recipes used for staining samples for electron microscopy are detailed in appendix three. Small, cubed samples of the papilloma ($\leq 1 \text{ mm}^3$) were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C overnight. They were then washed three times over a period of two hours in 0.1 M phosphate buffer at room temperature. Further fixation in 1% osmium tetroxide in 0.1 M phosphate buffer preceded serial dehydration in alcohol (50% ethanol 10 min, 70%, 95%, 100% x 2 for 20 min). The tissues were placed in 50% propylene oxide, 50% resin (Procure 812; Probing and Structure, Queensland, Australia) overnight (lid off). Tissues were then placed in fresh resin for six hours and polymerised for 48 hours at 60 °C in a final change of resin.

Thick sections (0.5 μm) were cut and examined to identify regions of the tissue at the outer border of the stratum spinosum. Thin sections (65 nm) were cut using an ultratome (Riechert-Jung Ultracut E). Thin sections were placed on 400 mesh copper grids and stained with saturated uranyl acetate in 50% ethanol (6 min). Grids were then washed with 50% ethanol, in distilled water, further stained with lead citrate (Sato, 1967) for six minutes and carefully dried before examination under an electron microscope (Phillips EM 201).

(ii) Preparation of papilloma for negative contrast EM : Discrete papillomas and tissue from the proliferative lesion were collected and minced finely with scalpel blades. Tissue fragments were ground to a fine paste with a minimum volume of PBS and sterile

sand in a mortar. The suspension was diluted to approximately 10% in PBS and centrifuged at $2800 \times g$ (30 min). Supernatant (4 mls) was placed onto a cushion (1 ml) of 45% (w/w) sucrose and centrifuged at $180,000 \times g$ for two hours (Beckman L8-70 ultracentrifuge). The pellet was resuspended in 50 μ l of distilled water overnight. Carbon-formvar coated grids were floated, coating side down (15 seconds), on a drop of 1% bovine serum albumin (BSA) solution placed on parafilm. The grids were transferred stepwise to a drop of the sample suspension (45 seconds), distilled water (15 seconds) and 2% phosphotungstic acid stain, pH 7.2 (30 seconds). The grids were blotted dry with soft tissue between steps and air dried before examination by electron microscope.

8.2.3 Transmission trial.

A possum, judged to be between 6 and 10 months of age, was tranquillised with ketamine and photographs of three intradermal inoculation sites recorded. The unfurred surface of the tail, the unfurred inner surface of the right pinna and the relatively lightly furred right inguinal areas were scarified with a hooked 18 g hypodermic needle that contained the 10% papillomavirus suspension. Trauma to the inoculation site was indicated by the presence of a raised welt and blood or tissue exudate. The possum was fed and housed in isolation for six weeks following inoculation.

8.2.4 Characterisation of papillomavirus DNA.

(i) Enzymatic digestion of papilloma : All reagents and recipes used in the molecular characterisation of the papillomavirus are detailed in appendix two. Material stored at -70°C from the papilloma was thawed. About 25 mg of the desiccated tissue was finely minced with scalpel blades, digested with PRETAQ™ (Gibco, BRL) and EGTA for 15 minutes at 75°C (Murray *et al.*, 1995) and an aliquot of the supernatant used in the PCR (see below). The same tissue was then further digested following a second protocol (Kawasaki, 1990) which was modified as described below. The tissue pellet was incubated for 1 hour at 55°C in four volumes (100 μ l total) of K buffer. A further 1 μ l of Proteinase K stock (10 $\mu\text{g}/\mu\text{l}$ in 10 mM Tris-Cl pH 7.5) was added and the digestion extended for 1 hour at 37°C . Proteinase K was inactivated by incubation at 95°C for 10 minutes and an aliquot stored at -20°C .

(ii) Polymerase Chain Reaction (PCR) amplification of papillomavirus DNA :

Published, human genital, PV specific, degenerate consensus PCR primers, for the PV L1 gene (Ting and Manos, 1990) were purchased (Gibco, BRL). Primers were resuspended in distilled H₂O to make a 100 μ M stock solution and stored (-20 °C).

MY09 (forward primer) : CGT CCM ARR GGA WAC TGA TC

MY11 (reverse primer) : GCM CAG GGW CAT AAY AAT GG

(iii) PCR reaction mix : The PCR reaction mix was prepared in a designated PCR room using dedicated pipettes, pipette tips and reagents. Samples were added to the reaction mixtures in a separate laboratory and the sealed tubes transported to the thermocycler in an adjacent room. A commercial PCR kit (Boehringer-Mannheim) supplied the reagents as a 10x cycling mix (containing 100 mM Tris HCl, 15 mM MgCl₂ and 500 mM KCl pH 8.3), a 10 mM dNTP stock (containing 10 mM each of dATP, dCTP, dGTP and dTTP) and Taq polymerase (5 units / μ l). The stock primer solutions were diluted to prepare 10 μ M working solutions and the reactions assembled, as shown below, with dH₂O to make a total volume of 100 μ l once samples were added.

10 μ l 10x PCR Buffer

5 μ l 10 μ M MY09

5 μ l 10 μ M MY11

2 μ l dNTP stock

0.5 μ l Taq Polymerase (2.5 units)

A master mix was routinely used to prepare multiple reactions. Taq polymerase was added last and all reagents held at 4 °C.

(iv) PCR conditions : PCR reactions were performed in 100 μ l thin-walled reaction tubes (Biotek) in a Perkin Elmer 9600 Thermocycler. Following an initial denaturation step of 94 °C for four minutes, DNA amplification was achieved using a repeating cycle consisting of 30 seconds denaturation (95 °C), 30 seconds annealing (55 °C) and one minute of extension (72 °C). When thirty amplification cycles were completed, reaction products were held at 4 °C or stored at -20 °C.

(v) Controls : Distilled water was always added as a negative control and a diluted aliquot of the original PCR product, kindly supplied by Joseph O'Keefe, was always used to confirm that the PCR was working. Normal possum skin and HeLa cells, incorporating human PV-18 DNA in a genomic background (kindly provided by Gail Meekin), were subject to the digestion procedure and served as further controls. The HeLa cell PCR product was subsequently purified and sequenced using identical methodology.

(vi) PCR products : Aliquots of reaction products were run on an agarose gel (1.5%) prepared with Tris/Borate/EDTA (TBE) buffer pH 8.0 containing ethidium bromide (EtBr) and electrophoresed with a molecular weight marker (Φ X174 RF DNA, *Hae*III fragments)(Gibco, BRL). The gel was run at 58 - 76 volts using standard electrophoresis equipment (Biorad), with TBE as the running buffer, for two hours. The gel was transilluminated with ultraviolet light at 312 nm (Spectroline^R Model TVC-312A) and a polaroid photographic record obtained (Sambrook, 1989).

(vii) GELaseTM purification of the PCR product : The PCR product was purified for sequencing using a GELaseTM (Epicentre Technologies) agarose gel-digesting protocol. To prepare DNA templates for sequencing, 40 μ l of the PCR reaction product was loaded into two wells of a 1% low melting point agarose gel (Seaplaque) made up with Tris/Acetate/EDTA (TAE) buffer, pH 8.5, containing EtBr as above, and run in TAE buffer for three hours at 58 volts. The product was cut from the gel, weighed in a tared 1.5 ml micro-centrifuge tube and melted at 70 °C in the presence of 1 μ l of 50x GELaseTM Buffer / 50 mg of gel slice (20 - 30 minutes). The gel slice was equilibrated to 45 °C, 1 unit of GELase / 300 mg of gel added and the mixture incubated for one hour. An equal volume of 5 M ammonium acetate and two volumes of absolute ethanol were added to precipitate the DNA which was pelleted by centrifugation (14,000 rpm x 30 min) at room temperature. The supernatant was removed, the pellet washed with 70% ethanol and centrifuged for 15 minutes. The supernatant was removed, the pellet dried for 15 minutes (Savant Speedvac SC100), resuspended in 50 μ l TE buffer (10 mM Tris Cl and 1 mM Edta pH 8.0) and stored at -20 °C.

(viii) Quantification of DNA for sequencing reaction : A 5 µl aliquot of the purified DNA was run on a 1.5% TBE gel (described above) against a DNA mass ladder (GIBCO, BRL) and the quantity of DNA estimated by direct comparison following UV transillumination.

(ix) Cycle sequencing of purified PCR product : Sequencing of the PCR product was achieved using the AmpliCycle Sequencing Kit (Perkin Elmer) as follows: Dideoxy nucleotide triphosphates (ddGTP, ddATP, ddTTP and ddCTP; G, A, T and C termination mixes) were dispensed (2 µl aliquots) into four PCR reaction tubes, one tube per termination mix. A master mix per primer was prepared (total volume 30 µl) and 6 µl was added to each termination mix (6 µl leftover) such that each sequencing reaction occurred in 8 µl total volume. The master mix contained 20 µM primer (either MY09 or MY11) and 30 ng of template DNA, 10x cycling mix (containing ordinary deoxy nucleotide triphosphates, thermostable polymerase and appropriate ionic conditions) and [^{33}P] dCTP radioactive label. The volume of autoclaved dH₂O was adjusted as a function of the volume and concentration of the other reactants in the master mix. The label was added last, after the addition of the cycling mix and all tubes kept on ice at 4 °C whilst the reactions were assembled.

(x) Cycle sequencing conditions : Cycle sequencing, Perkin Elmer (GeneAmp PCR System 9600), began with a denaturation at 95 °C for two minutes. The cycle proper comprised a denaturation step at 95 °C for 30 seconds, annealing at 65 °C for 30 seconds and extension at 72 °C for one minute. Twenty five cycles were completed and the reactions held for a minimum period at 4 °C prior to the addition of 4 µl of the stop solution (95% formamide, 0.05% Bromophenol blue, 0.02% Xylene Cyanole FF in 20 mM EDTA). The reactions were mixed with the stop solution and stored (-20 °C).

(xi) Preparation of the sequencing gel : A standard 6% denaturing polyacrylamide urea sequencing gel was prepared following standard protocols (Sambrook, 1989) in accordance with the manufacturers instructions (Life Technologies Sequencing Gel Electrophoresis System : Model S2 #1105). Glass plates used to support the gel were thoroughly cleaned and detergent residues removed with deionised water.

A final clean with ethanol and application of silicon to the shorter plate preceded assembly and sealing of the plates. Air bubbles were eliminated during gel pouring.

(xii) Running the cycle sequencing products : The sequencing gel was placed in the running apparatus (BRL Life Technologies model S2) and pre-electrophoresed for 30 minutes (70 watts constant). Samples from the cycle sequencing procedure were denatured at 94 °C for 3 minutes and transferred to ice at 4 °C. Wells were flushed with TBE buffer prior to loading 3 µl of each sample in a G A T C configuration. Both long (6 hours) short (3.5 hours) and very short runs (1.5 hours) were performed. Denaturing and flushing steps were repeated prior to each loading.

(xiii) Preparation of gel for autoradiography : The gel apparatus was disassembled and the shorter glass plate prised off the gel. Gel fixative (5% methanol / 5% acetic acid) was gently poured onto the surface of the gel and left for 15 minutes. Wrinkles were eliminated, the gel transferred to 3MM Whatman filter paper and dried for 30 minutes (BIO-RAD Model 583 Gel Dryer). Following drying, the gel was checked for radioactive emission (Series 900 Mini-Monitor G-M Tube; Mini Instruments Ltd) and placed in direct contact overnight with Xray film in a cassette containing a single intensifying screen. The Xray film was developed and the sequence read manually (Ausubel *et al.*, 1995).

(xiv) Alignment of papillomavirus sequence : Sequence information derived from gel autoradiography was entered into GENWORKS version 2.45 (IntelliGenetics, Inc) and aligned. Papillomavirus DNA from the digestion performed by Meekin and co-workers was purified and sequenced concurrently with material stored at Massey University. Sequence data from the Meekin material was aligned with the Massey material to provide further support for the novel papillomavirus.

(xv) Comparison of the PPV sequence with NCBI database submissions : The aligned and edited papillomavirus sequence was entered and sent for comparison with DNA sequences in the NCBI database. This comprised non-redundant GenBank + EMBL + DDBJ + PDB sequences, representing >246,000 sequences and >346,000,000

letters (July, 1996). The format followed was as for a BLAST (Basic Local Alignment Search Tool) search with the sequence information conveyed from the database to the requester via the internet. The default settings in the search program were chosen and 100 homologous sequences were reported. The translated papillomavirus DNA sequence was also submitted to the NCBI database as an amino acid sequence. This comprised non-redundant GenBank CDS translations + PDB + SwissProt + SPupdate + PIR sequences, representing >302,073 sequences and >91,112,663 letters (May, 1998). A selected, representative subset of the returned amino acid sequences were aligned with the possum amino acid sequence using highly conserved flanking motifs. Pairwise comparisons of each edited sequence with the possum amino acid sequence were performed using GENEWORKS (as above).

8.2.5 Comparison of selected papillomavirus sequences : Phylogenetic analysis.

Fifteen representative human and animal PVs were chosen in order to construct a PV phylogeny that contained previously published PV diversity (Chan *et al.*, 1992, 1995, 1997 and 1997a) and included the possum sequence. These PVs included human genital and epidermodysplasia verruciformis (EV) PVs, genital and EV PV sequences from colobus monkeys, a variety of non-primate animal PVs and an avian (chaffinch) PV (Table 8.3 tessellated boxes). The L1 gene for each papillomavirus of interest was obtained as an amino acid sequence from GenBank (NCBI, Bethesda, Md. USA) or translated from the deposited nucleotide sequence. Amino acid sequences (~150 amino acid residues amplified by the MY09 and MY11 primer pair) were aligned using Clustal W version 1.5. Subsets of approximately 100 and 127 aligned amino acid residues were used for tree building. The tree-like properties of the data were first examined using Split Decomposition, version 2.4 (Huson, 1998) to investigate the 15 taxon case. The resulting splitsgraph clearly indicated that the data would support a tree-like phylogeny. The ovine and chaffinch taxa were removed when the splitsgraph indicated they would probably not contribute to resolution of PV relationships amongst the other chosen PVs and the data was then reexamined using a more sensitive character state approach (parsimony) to build a papillomavirus phylogeny (PAUP 3.1.1). Bootstrapping was carried out to evaluate the relative number of patterns in the data supporting particular groupings of taxa. Trees produced from 12 and 13 taxon cases, without and including

the sequence of interest respectively, and then following other sequence omissions and additions were analysed and tested for stability. A further tree was reconstructed using 14 taxa following omission of only the chaffinch sequence. This omission enhanced alignment of a variable (phylogenetically useful) portion of the L1 gene and 127 peptides were included in a similar phylogenetic analysis using these data.

8.3 RESULTS

8.3.1 Anatomy and histopathology.

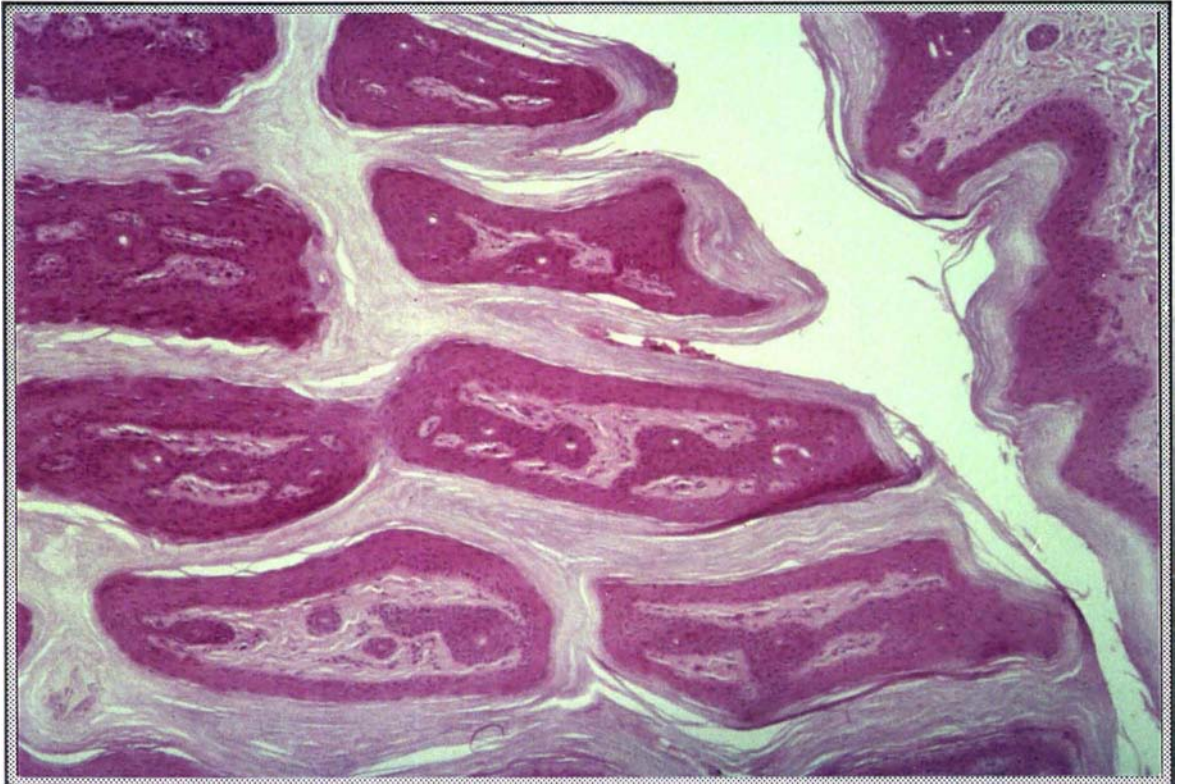
Discrete, circular, wart-like lesions (4-10 mm in diameter) projected one millimetre or less from the ventral surface of the tail. An irregular, proliferative hyperkeratotic plaque (30 mm x 8 mm) projected 1-3 mm from the ventral tail surface and appeared to be formed from small coalescing warts. As this site was altered and partially obscured by scarring and serum exudation following biopsy respectively, the anatomy of the gross papillomatous changes were not clear. At the junction of the furred and non furred areas, epidermal proliferation was replaced by crusts and fragments of exfoliating epidermis. Wart-like lesions at the distal end of the tail had partially regressed at submission.

Histologically, the squamous epithelium and dermis were disrupted by abnormal proliferating keratinocytes and hyperkeratosis (figure 8.1). The basal layer was less organised than adjacent normal epidermis and extended into numerous dermal pegs (0.2 - 0.4 mm diameter) supporting a hypercellular stratum spinosum, composed of irregularly arranged cells with large nuclei (Figure 8.2). The proliferating stratum spinosum of one dermal peg fused with that of another to form a thickened mass of disorganised, poorly differentiated keratinocytes. Proliferating dermal pegs were supported in an even keratin matrix following the contour of the individual pegs. Irregular cells, keratohyaline granules and small keratin whorls were identified within the centre of the dermal pegs (figure 8.3). The lesion did not contain a significant fibrous element or suggestion of malignant progression.

8.3.2 Electron microscopy.

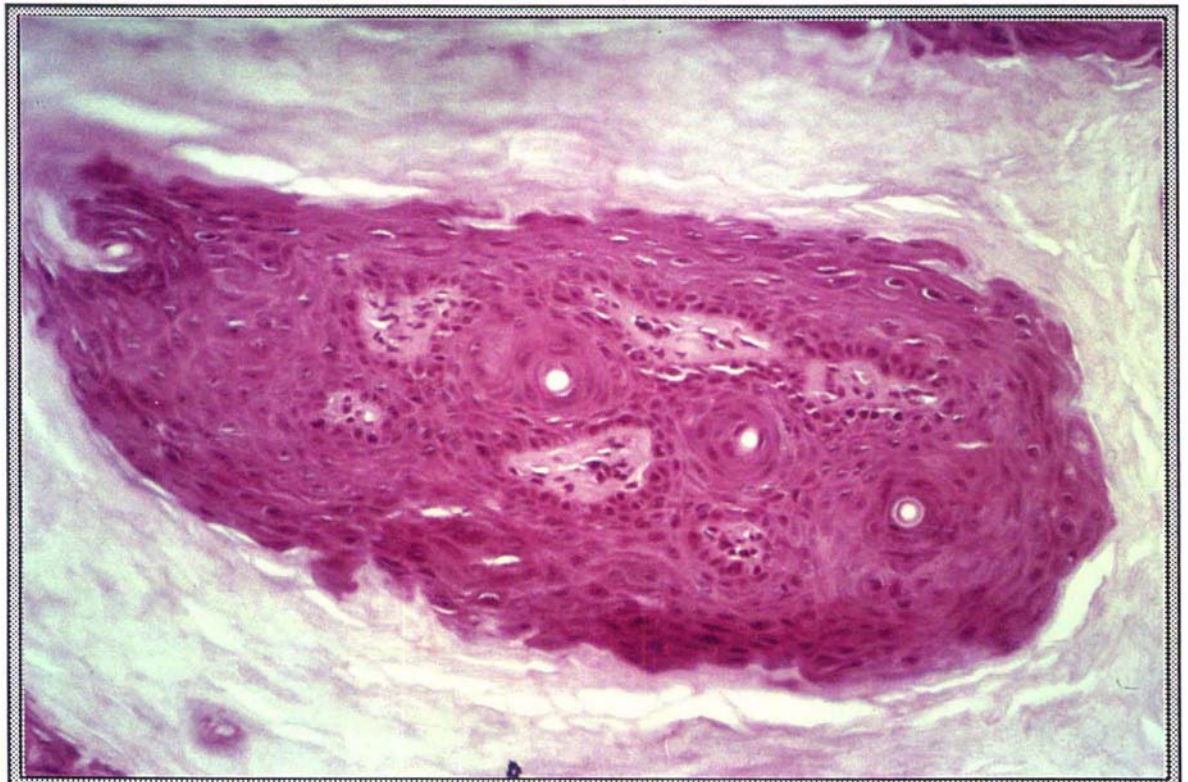
Virus particles were observed in most fields at medium magnification (70,000 x) when the disrupted and negatively stained preparation was viewed. At a magnification of 207,000 x, the particles were demonstrated to have a diameter of 55 nm (figure 8.4). Capsomeres were discernible and an overall spherical appearance was slightly suggestive of an underlying icosahedral symmetry (Figure 8.4). No viral particles or intranuclear inclusions were demonstrated within resin embedded thin sections.

Figure 8.1 Possum papilloma : H & E stain (x 86).



The papilloma was composed of semi-regular dermal pegs, seen as discrete elongated intrusions into the dermis, adjacent to the normal epithelium (upper right).

Figure 8.2 Possum papilloma : H & E stain (x 215).



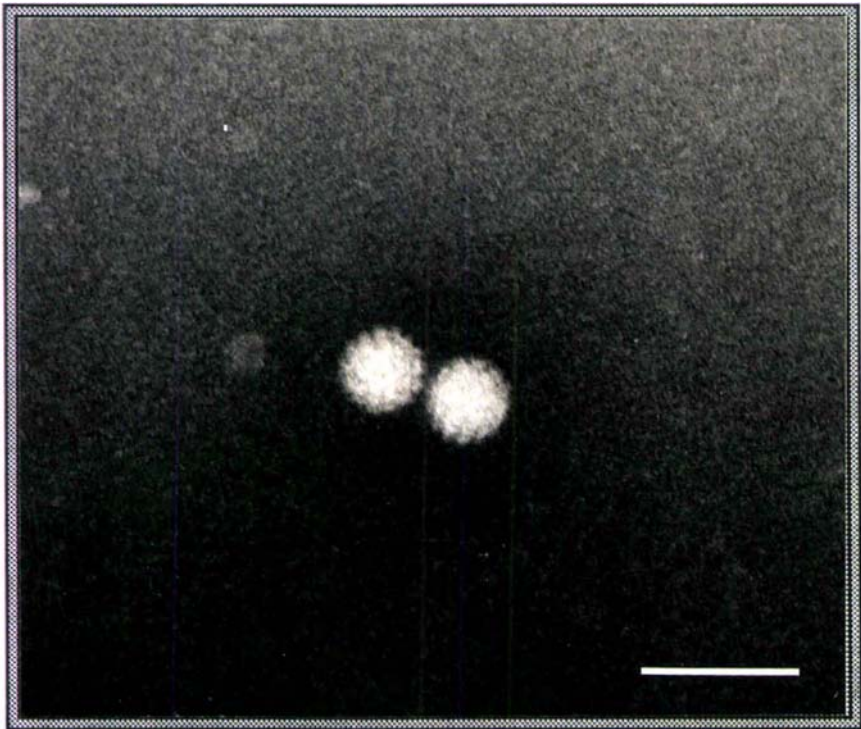
Increased cellularity of the basal layer and stratum spinosum seen together with fusion of dermal and epidermal elements.

Figure 8.3 Possum papilloma : H & E stain (x 860).



Irregular cells, keratohyaline granules and small keratin whorls were identified within the centre of the dermal pegs.

Figure 8.4 Negatively stained papillomavirus particles : 207,000 x magnification.

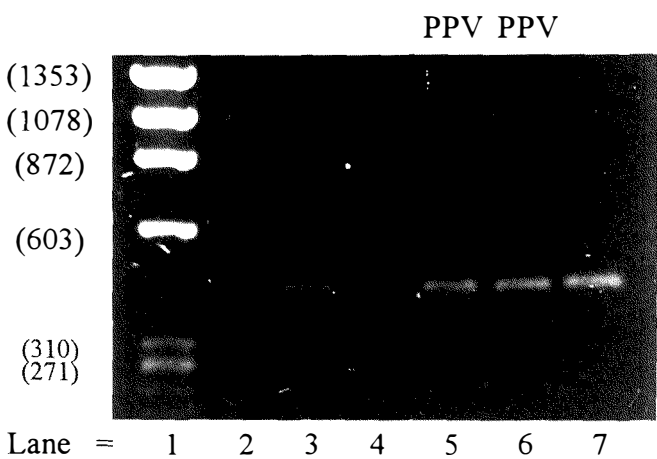


Papillomavirus particles (~55 nm diameter) are shown with an overall spherical appearance and underlying icosahedral symmetry. Bar represents 100 nm.

8.3.3 Transmission trial.

No reaction was observed following inoculation in the groin or on the tail. A fine scab developed at the site of scarification on the inner surface of the ear. Over a three week period this lesion developed into a thin crust on the skin surface but spread more laterally than the initial scarification. At four weeks post-inoculation the lesion began to regress and the scab was removed for investigation by E.M. There was nothing abnormal noted in the dermis underlying the scab and no virus particles were identified by E.M. At six weeks post-inoculation the site could not be easily distinguished from surrounding tissue and the experiment was terminated. Skin samples from the original inoculation sites were not investigated histologically or using molecular biological procedures.

Figure 8.5 Amplification products from possum papillomavirus DNA.



Lane 1 = Φ X174 RF DNA, *Hae*III fragments (molecular weight marker : base pairs).
 Lane 3 = PPV tail digest : Papillomavirus DNA in genomic background (Gail Meekin)
 Lane 4 = dH₂O (negative control)
 Lane 5 = PPV tail digest : Protocol described above: 8.2.4 (i)
 Lane 6 = PPV tail digest : 20 μ l sample prior to second digestion : 8.2.4 (i)
 Lane 7 = HeLa cell digest : Papillomavirus DNA in genomic background; Gail Meekin positive control)

8.3.4 Papillomavirus PCR.

Following electrophoresis of PCR products, a DNA band approximately 450 bases in length was identified (Lanes 5 and 6 : labelled PPV in figure 8.5). The electrophoretic migration of these DNA species in agarose was identical to that of the HeLa cell digest (Lane 7 : positive control) and other papillomavirus containing samples (Lane 3). Lane two contained the same template (PPV tail digest) as lane three but a slightly less than

optimal volume of the master mix (affecting ionic conditions) probably prevented amplification in this lane. No amplification was obtained in any negative controls. The amount of DNA in 5 µl of the GELase™ purified PCR product was estimated to be 25 - 30 ng (data not shown).

8.3.5 Sequence data.

Approximately 150 bases could be read from each gel for the long and short runs. The very short runs produced approximately 100 readable bases with minor ambiguity for a few bases within the non-overlapping readable sequence (< 6 bases). Sequence information from the Meekin papillomavirus L1 gene was identical and all available data was entered into GENEWORKS in order to form the overlapping and continuous 393 base pair sequence (Table 8.1). The HeLa cell positive control sequence was visually different to the PPV material and 165 bases from this former sequence was completely homologous to human papillomavirus (HPV) -18.

Table 8.1 Possum Papillomavirus DNA Sequence (393 bp).

TGACATACGT	TCGATAGATC	AACATTCCA	AAGTTTAACT
TTTCATAAGG	ATCCACCTTG	TCCGTAGGGG	GATTAGCATC
AGGGCATCGG	GTTGCAAGGG	AGTTAATGAA	GCGGTACATA
TCTTCTAGGG	GATTGTTAGG	AGTGGGTACA	AATCCTAAAT
TCCAAGAATC	CAGTATAAGG	GGATTCATAG	CATTAATATG
TGAAAGTACA	TCAGACTCCA	GAGGGACCTT	GCAAAGTTGG
AATATAAAGG	ACAACTCTAT	TTCTCTACA	TGACGGAGAT
ACTGCTTATA	GTTATCATTG	TTAAATGTTT	GTGGTGGGGG
GTCAACATCT	AATACAGAGA	TGGAGAAATT	ATAGTTGCGT
GTATTATCAA	CCATTGTCAG	GAATAGCTCA	TTG

8.3.6 Comparison with database sequences.

The DNA sequence information obtained from the tail lesion on the possum revealed matches with human and animal PV sequences. The most similar genomes to the possum PV came from HPVs of the EV supergroup. Types 49, 25, 36, 37, 38, 5b and 8 were among 16 of these supergroup B viruses that were identified in the BLAST search. Human PVs from this supergroup shared between 58% and 69% identity with the

possum PV sequence (Table 8.2). Human PVs 70 and 39 from supergroup A (genital HPVs) shared 55% identity with the presumed possum PV. The pygmy chimpanzee PV and bovine PV type-4 shared 59% and 58% identity with the possum PV respectively. The similarity of the HPVs from supergroup B to each other was investigated by submitting the sequence for the L1 gene of HPV-25 to the NCBI database as described above. The results of this search identified HPVs from the same supergroup. The three nearest relatives to HPV-25 at a genome level had greater than 80% identity and the nearest dozen had greater than 70% identity.

Table 8.2 Possum PV : Nucleotide comparison with other PVs.

PAPILLOMAVIRUS	PPV HOMOLOGY
Human PV supergroup B (EV-HPV)	58 - 69 %
Pygmy chimpanzee PV	59 %
Bovine PV type 4	58 %
Human PV supergroup A (genital HPVs)	55 %
Bovine PV type 5	52 %
EV-HPVs (Supergroup B)	EV-HPVs (type 25)
HPV-25	70 - 84 %

PV - papillomavirus PV - Human papillomavirus EV - epidermodysplasia verruciformis

The amino acid sequence identity showed the same pattern of relationships (table 8.3). Human EV PVs were the most similar to the possum PV sequence with 56 - 62 % of amino acid residues being identical. Many non-identical amino acids were functionally related to the corresponding residue in the possum sequence.

Colobus monkey PV-2 (CgPV-2) was as similar to the PPV as many other human EV PVs on the basis of amino acid identity. Human genital and primate papilloma viruses (including genital strains) shared 44 - 48% homology with the PPV sequence. The multimammate mouse PV, bovine PV-4 and the canine oral PV (each host in a separate mammalian order) had 51, 50 and 47% identity respectively (greater than or equal to the identity of the human genital and primate PVs with the PPV). Other animal PVs and the chaffinch PV were less similar.

Table 8.3 Similarity of possum PV amino acid sequence to other PV sequences.

Papillomavirus type ¹	Homology (%) ²	+ VE ³	Anatomical site of lesion
HPV-19	62	9	Skin (EV super group)
HPV-25	59	6	Skin (EV super group)
HPV-47	58	9	Skin (EV super group)
HPV-17	57	8	Skin (EV super group)
HPV-8	57	10	Skin (EV super group)
HPV-37	56	7	Skin (EV super group)
HPV-38	56	9	Skin (EV super group)
HPV-49	56	8	Skin (EV super group)
CGPV-2	56	9	Skin (EV super group)
HPV-36	55	12	Skin (EV super group)
Macaca PV	48	11	Genital mucosa
HPV-39	48	6	Genital mucosa
HPV-18	47	8	Genital mucosa
HPV-70	46	10	Genital mucosa
HPV-11	44	13	Genital mucosa
Common chimp PV	47	12	Oral mucous membranes
Pygmy Chimp PV	45	12	Genital mucosa
Rhesus monkey PV	45	12	Genital mucosa
CGPV-1	44	7	Genital mucosa
Multimammate Mouse PV	51	9	Skin
Bovine-PV-4	50	10	Alimentary tract
Canine oral PV	47	9	Skin / oral mucosa / gingiva
Cotton-tail rabbit PV	43	7	Skin
Ovine-PV	43	5	Skin
Bovine-PV-1	40	8	Skin
Chaffinch	37	4	Skin

1. Papillomavirus types in tessellated boxes were used to construct a papillomavirus phylogeny.

2. Percentage of amino acid residues from possum PV sequence having identity (homology) with the corresponding (aligned) amino acid residue from other selected PV sequences.

3. + VE = Positives. Number of non-identical but functionally related amino acid residues.

8.3.7 Phylogenetic analysis of sequence data.

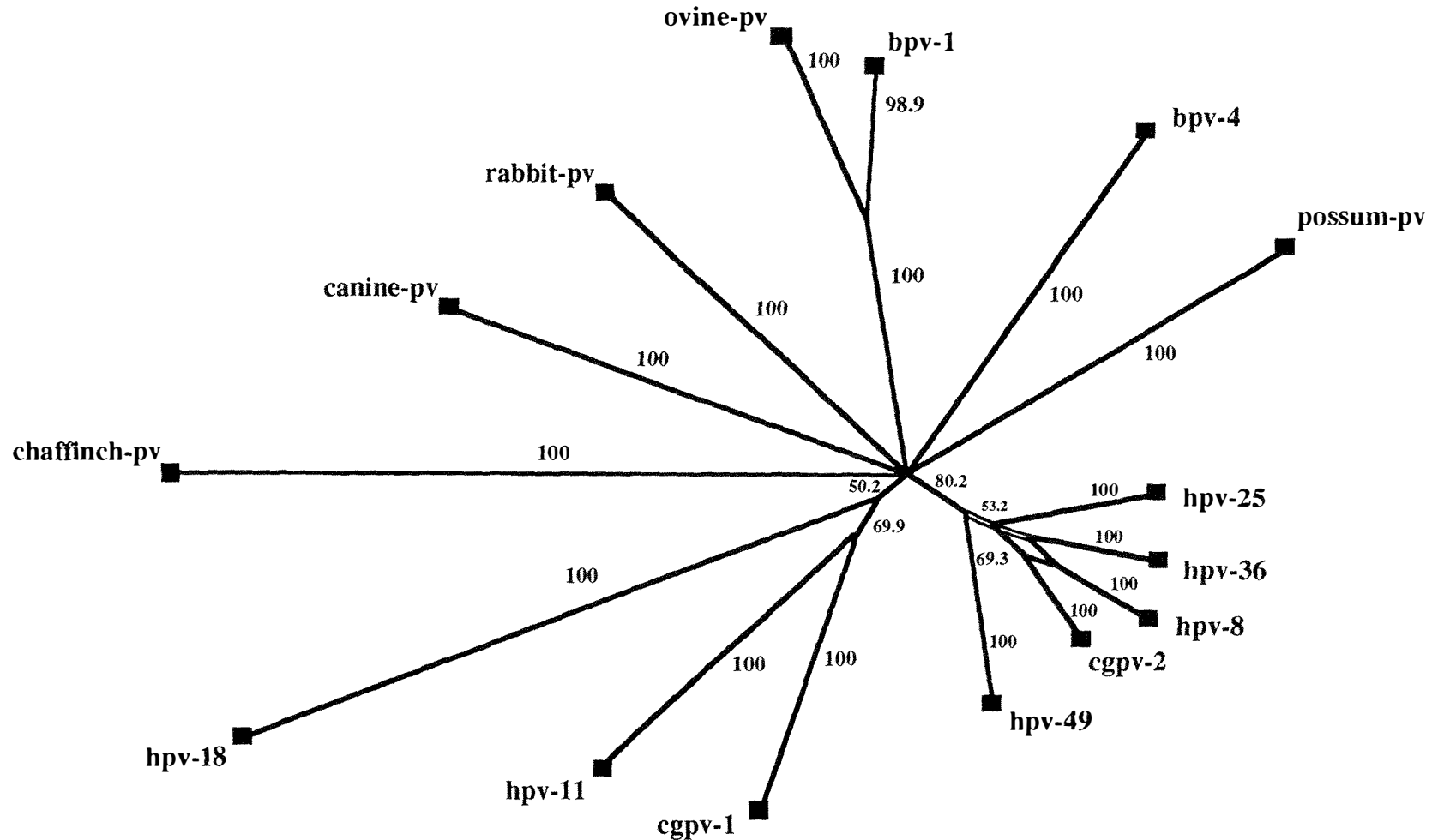
Split decomposition demonstrated the tree-like nature of the data (figure 8.6). In this structure, primate EV PVs were separated from the genital PVs. Animal PVs were separated from both groups. The possum PV was no more similar to the EV PVs or the genital PVs than several of the other animal PVs. This was in contrast to the results from PAUP analysis in which a phylogeny within the animal PVs was suggested.

Three strong trends were demonstrated following the reconstruction of a further papillomavirus phylogeny using parsimony (PAUP 3.1.1). These trends were maintained throughout subsequent rearrangements of taxa to test the stability of proposed evolutionary relationships (figure 8.7).

1. Primate EV PVs were consistently grouped together (bootstrap values 78 - 100).
2. Primate genital PVs were consistently grouped together and distinct from all other PVs (bootstrap values 72 - 85).
3. Animal PVs (cutaneous and alimentary species) were consistently more similar to the EV PVs (cutaneous) than to genital PVs. Bootstrap values for separation of the animal PVs from one another and from the EV PVs varied (bootstrap value range 34 - 100).

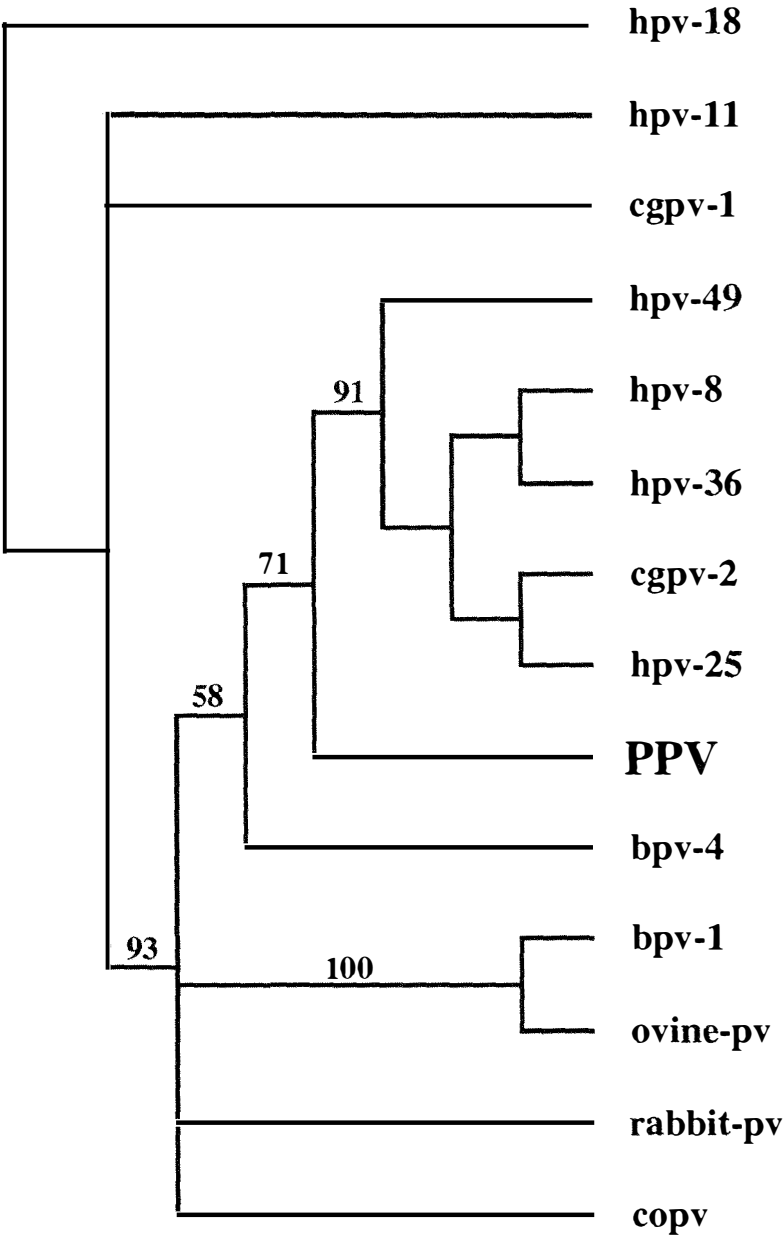
Addition of the possum PV sequence did not change the tree topology. Neither omission nor addition of taxa altered tree shape but the associated bootstrap values supporting various groupings of taxa were altered. Realignment of the taxa following omission of the chaffinch sequence, enabled more sequence information to be retained in the analysis. Tree topology was constant. There was 91% support from bootstrap values that the EV PV sequences were split from all other sequences even though the closest relative was the possum PV. There was a maximum of 3% support in the data for grouping the PPV within the EV-PV group.

Figure 8.6 Splitsgraph of phylogenetic relationships between 15 papillomaviruses



The tree-like nature of the data are indicated by the bifurcating splitsgraph above. As a preliminary PV phylogeny it suggests that the human EV-PVs form a distinct clade (80.2% of data groupings support this split). There is also moderate support for a human genital PV branch in this phylogeny (50.2% support). Animal PV sequences, including the possum sequence, were distinct and emerged from a point between the EV and genital HPVs (in a splitsgraph, taxa may rotate about a point origin; the closest neighbours to the possum and chaffinch PVs are not necessarily the EV-HPVs or genital HPV groups respectively). With the exception of the ovine sequence and BPV-1, no relationships amongst the animal PV sequences were suggested and all other taxa, including the human PV sequences, were considered to be distinct.

Figure 8.7 Phylogenetic relationships between 14 papillomaviruses (PAUP 3.1.1).



Unrooted and unweighted tree produced from 14 taxa on a 127 amino acid alignment using parsimony. Strong splits in the data were those identified under split decomposition and include the EV HPVs (91% bootstrap support) and genital HPVs (93% bootstrap support). In contrast with the (conservative) splitsgraph above, some phylogenetic signals amongst the animal papillomaviruses are resolved. Bootstrap values supporting some of these splits are given.

8.5 DISCUSSION

As papillomaviruses are normally highly species-specific, papillomavirus particles demonstrated in a typical cutaneous papilloma on the tail of a possum suggested a new papillomavirus type. However, in spite of much accumulated knowledge that shows papillomaviruses to be host-specific, and often site-restricted within a host, there are notable exceptions in the veterinary literature. Bovine papillomavirus DNA was found in naturally occurring equine sarcoids (Amtmann *et al.*, 1980; Bloch *et al.*, 1994a; Otten *et al.*, 1993) and both BPV-1 and -2 produce fibrous tumours in hamsters and mice (Olson, 1987). While these cases present strong evidence that some papillomaviruses can cross species boundaries to produce fibrous tumours, the lesion in rodents was unnaturally acquired and non-productive (Gross, 1970). Productive papillomavirus infection has been difficult to demonstrate in equine sarcoids and horses with naturally occurring sarcoids lack antibodies against bovine papillomavirus in comparison with horses experimentally infected with bovine papillomavirus and producing fibromas resembling sarcoids. None-the-less, the horse has been considered as a non-permissive host for bovine papillomavirus infection (Vanselow and Spradbrow, 1996).

Evidence for cross-species transmission was provided when cottontail rabbit papillomavirus (CRPV) induced productive infections in domestic rabbits, jack rabbits, snowshoe hares and rats following experimental inoculation of CRPV (Wettstein, 1987). The amount of CRPV produced varied both with lagomorph host and strain of CRPV used. While only cottontail rabbits produced CRVP efficiently, domestic rabbits were equally, if not more, susceptible to the oncogenic effects of CRVP infection.

Evidence for zoonotic cutaneous papillomatosis was identified as a high incidence of the rare HPV-7 in slaughterhouse workers handling raw meat (Keefe *et al.*, 1994; Melchers, 1993; Stehr-Green *et al.*, 1993). Although HPV-7 has not yet been identified in animal tissue, neither wear and tear to hands nor wet and cold working conditions were enough to increase the attack rate in occupations where there was no exposure to meat (Keefe *et al.*, 1994; Stehr-Green *et al.*, 1993). In the absence of HPV-7 in meat, an as yet unidentified cofactor in animal flesh facilitating the establishment of warts remains a plausible explanation (Keefe *et al.*, 1994).

While most available evidence indicates strong host specificity for cutaneous papillomaviruses it seemed coincidental that the first observation of papillomas in a possum should occur on an individual animal which had been frequently handled. Therefore, it was important to characterise this virus as fully as possible to determine whether or not it was a cross infection from another species.

One convincing piece of evidence for a possum adapted PV would have come from a positive result to the transmission trial. Unfortunately, the transmission experiment did not result in the development of a detectable papilloma in the recipient possum. This finding was similar to the reported failure to propagate the opossum papillomavirus (Koller, 1972). Clinical material from the sites of inoculation was not saved for further investigation, which may have been able to detect the presence of PPV DNA in a quiescent state or early gene products prior to productive infection. Papillomatous samples were extremely limited and rather than risk the unique genetic material in further transmission experiments it was decided to save the remaining starting material in order that the entire genome could be cloned in future research.

In order to test the hypothesis that the PPV is both unique and specific to possums, it was necessary to examine the phylogenetic relationship between PPV and EV HPVs. If PPV has a long history in the marsupial lineage, then the expectation would be that the sequence should be distinct from the human EV-PVs. The closest PV, a human EV-PV (HPV-8), had 69% DNA sequence identity with the PPV. Two other human EV-PVs, HPV-17 and HPV-15, had 58% and 59% DNA sequence identity with the PPV respectively. Human EV-PVs were more closely related to one another than to the PPV, favouring the hypothesis that the possum PV sequence represents a distinct lineage with a long history in a marsupial ancestor.

The degree of identity or similarity between human EV-PVs and the PPV may reflect parallel or convergent adaptation to similar ecological niches. In support of this contention, we observe that BPV-4 and pygmy chimpanzee PV, which replicate in non-keratinized, stratified, squamous epithelia of the upper digestive tract and oral cavity respectively, also show a high level of sequence identity with the PPV (58% and 59%

respectively). In contrast, human supergroup A PVs, which have a predilection for the genital tract, showed less similarity to the PPV (55%) than the animal PVs mentioned above. Similar relationships apply with the amino acid identity data in a greater range of animal species (Table 8.3). For example, the possum PV showed the same level of similarity to colobus monkey PV-2 (EV-PV) as it did to human EV-PV types and was less related (44 - 48%) to a range of primate genital PVs. The evolution in the genital PV sequences may show a distinct pattern of substitution when compared to EV PV sequences, possibly as a result of differing constraints on PV sequences in the genital environment. These data support a hypothesis that the possum PV is more similar to cutaneous PVs in general than to genital PVs and is not specifically similar to human PVs even though its nearest neighbours are the human EV-PVs.

Hypotheses suggesting convergent protein evolution have been made elsewhere and strongly supported using molecular phylogenetic analysis (Swanson *et al.*, 1991). In one study it was observed that distantly related stomach lysozyme genes from cows and langur monkeys have (presumably) evolved in similar environments of foregut fermentation. It is thought that this has led to the parallel substitution of identical amino acids in both sequences, since such a degree of similarity is not reflected in comparisons of neutral molecules (Non-coding regions of the gene and silent (third base) positions in codons).

While hypotheses regarding evolutionary relationships may be inferred from dissimilarity data alone, tree building methods detect a greater range of spatial patterns in the data. In the present study they provide robust support that the possum PV is distinct from its nearest neighbours. The strength of the phylogenetic signal in the data (as measured by bootstrap analysis, figure 8.7) supports the hypothesis for a long history of independent, parallel cutaneous evolution. With the bootstrap resampling, PPV joins with an EV-PV in 3% or fewer of the resampled data sets. Further, stability of the tree was tested by including or omitting certain taxa. Whilst this approach showed some increase or decrease in the bootstrap support for separating the possum PV from EV-PVs in reconstructed trees, greatest support always favoured the former as a distinct lineage.

Although fewer than 400 bp were studied in the present case, the bootstrap analysis suggests that conclusions would not change even if larger sequences were available for the same loci. Clearly, the hypothesis which is favoured in any analysis might be tested further by examining alternative regions of the possum PV genome. However, Chan *et al.* (1992, 1995) have also reported that other loci (E1, E6 and E7) in papillomavirus genomes support similar hypotheses amongst the PV types included in their studies. This result gives confidence in the findings of the present study.

Papillomavirus evolution is proposed to have predated the evolutionary split between monkeys and apes (Chan *et al.*, 1997a). This contention is supported by the identification of genital PVs and EV PVs in the colobus monkey and demonstration of their similarity to human PVs which occupy the same host sites (Chan *et al.*, 1997). Assuming this well studied model of virus evolution is correct, it is feasible to suggest that the ancestral cutaneous PV has evolved in possums since speciation and that sequence divergence from human as well as a number of animal PVs has been relatively constrained by the need to maintain productive virus infections in similar, yet highly differentiated, cutaneous locations. In this situation the constraints applied by differentiating squamous epithelia would impose a measure of convergent evolution on viruses that have been evolving independently for millennia and a coincidental high level of similarity between possum and human EV PVs does not imply cross-species transmission.

Ecology of possum papillomavirus : Cutaneous papillomas frequently occur in sites exposed to wear and tear. Human warts occur most commonly on the hands although lesions in all external locations have been recorded (Grußendorf-Conen, 1987). In cattle, the sites of predilection are the neck, shoulder, chin, udder and dewlap areas which are subject to abrasions and wounding (Olson, 1987) with dairy cattle displaying a variety of papillomatous lesions (and papillomavirus types) on teats (Meischke, 1979). Possums use their prehensile tails as an extra limb when climbing and balancing in trees and thus this site is predisposed to natural wear and tear. Other cutaneous sites that are directly exposed are the lightly furred areas of the head, feet and pouch and it is likely that these may be areas predisposed to papillomavirus infection. In observing the temporal events

of cutaneous papillomatosis in opossums it was suggested that the feet and pinnae were natural primary and secondary sites respectively for papillomavirus infection (Koller, 1972).

Papillomaviruses resist acids, lipid solvents, freezing for several months (-25 to -35 °C) and heating (50 °C for 1 hour)(Gross, 1970; Murphy *et al.*, 1995). Although the length of time PVs retain infectivity in nature is unknown (Grußendorf-Conen, 1987), it is hypothesised that PVs survive for considerable periods in the environment or on fomites. Transmission is widely believed to require some degree of trauma to an epithelial surface for the virus to gain access to basal cells (Grußendorf-Conen, 1987). Predictions about the deposition and survival of the possum papillomavirus in the N.Z. environment can be made from our knowledge of the biology of other papillomaviruses. Ambient conditions found in N.Z. forests and other possum habitats for many months of the year would favour survival of these non-enveloped viruses off the host. Possums using the same habitats could transfer the virus passively in den sites and on trees that are preferentially browsed. Agonistic interactions between possums in areas that are densely populated, during the mating season or during dispersal of young animals could provide another mechanism for virus transmission.

That these relatively characteristic lesions have not been previously described in the N.Z. possum population, which has both been intensively studied recently and extensively hunted for commercial and control purposes for several decades, is surprising. From a single identification, in the absence of reports of typical papillomas in possums, it can only be speculated that the prevalence of PPV in the New Zealand possum population is low.

By analogy with other host species, cutaneous papillomaviruses infect young animals for medium periods of time with immune competent animals rejecting the papillomas after a few months. Epidemics of cutaneous papillomatosis are not uncommon and usually involve young cohorts in a confined area. Examples of this include observations of bovine warts by Bagdonas and Olson (cited in Olson, 1987) and the distinct rise in prevalence of human warts in school age children (Grußendorf-Conen, 1987). However,

even in areas of high possum population density, the level of contact between possums would be likely to fall below that of other animal species that are confined.

Biological Control : Absolute host specificity is one of the most important requirements for agents being considered for biological control purposes. Papillomaviruses generally fit this requirement and, as the survey sought to identify viruses meeting this criterion, the suitability of papillomaviruses for use as a self-perpetuating pathogen and/or vector for biological control was considered. Papillomaviruses are not highly contagious in the absence of close husbandry situations and although they are reasonably resistant in the environment, some estimates for the likely transmissibility of the PPV in natural New Zealand environments would be required.

When considering the potential use of a papillomavirus vector as an immuno-contraceptive for possums (Tyndale-Biscoe, 1991), the low grade immune response to papillomaviruses must be regarded as a negative factor. Humoral responses to cutaneous papillomavirus infection often develop slowly and may be stronger to early papillomavirus gene products than the peripherally located, structural gene products (Tindle and Frazer, 1994). The immune response against a given possum antigen central to successful reproduction, may rely on humoral responses, cell-mediated phenomena or both. Characterisation of the possum immune response to PPV infection may help to predict where addition of DNA (encoding immunogenic peptides) to create an immuno-contraceptive vector (genetically modified PPV) may most effectively stimulate the appropriate immune response. For example, if an effective humoral response was required, new genes to be transcribed would need to be under the control of promoters controlling early gene expression. If a cell-mediated response was required to effect infertility then the strategy employed by papillomavirus with respect to interaction with the host immune system may be a suitable model for further investigation.

Unfortunately serological research with papillomaviruses is still hampered by the lack of *in vitro* culture systems. For example, the lack of standard neutralisation assays prevents neutralising antibodies in convalescent sera from being assumed or measured. The

difficulties of obtaining high quality sero-epidemiological data from populations infected with papillomaviruses has been highlighted in a review article (Spradbrow, 1987) and these constraints would apply to assessment of PPV prevalence in New Zealand possums.

At a technical level the DNA manipulations required to make recombinant papillomaviruses have been established (DiMaio, 1987) but production of infectious recombinants from culture systems is still hampered by the general difficulty of papillomavirus propagation *in vitro*. The small genome restricts the amount of recombinant DNA that could be added and physically encapsidated and there is only a small proportion of the genome that is able to be replaced. None-the-less there is sufficient flexibility for foreign proteins to be expressed even if the recombinant insert is limited to a few important epitopes of essential possum proteins. Furthermore, several specialised systems are available for the study of papillomavirus propagation *in vitro* and could be applied to a clear set of objectives (Meyers and Laimins, 1994; Kuo *et al.*, 1994; Mungal *et al.*, 1992; DiLorenzo *et al.*, 1992; Bossens *et al.*, 1992).

Further work on the possum papillomavirus must conserve the small amount of clinical material remaining. Only when the entire papillomavirus genome has been cloned and preserved, should full length copies be made available for further investigation *in vivo*. Then follows research to determine ease of artificial transmission, confirmation of host range, site specificity, age susceptibility, duration of infection, nature of immune response and ease of natural transmission. If another live captured possum with papillomas is identified, it would be worth determining whether or not infection could be transmitted by exposing young (presumed) susceptible possums to the infected individual in a group housing situation.

8.6 SUMMARY

A naturally occurring case of cutaneous warts in a New Zealand possum was investigated. Typical papillomas were circular, raised, hyperkeratotic and histologically identical to non-fibrous papillomas of other species. Proliferative and disorganised dermal pegs interrupted the normal epithelium. Thickened squamous epithelium and associated increase in keratin were distinctive features. Papillomavirus particles were demonstrated by EM in appropriately prepared suspensions from the papilloma.

Papillomavirus DNA from the conserved L1 gene was amplified by PCR (consensus papillomavirus specific primers), purified and manually sequenced. Comparison of 393 bases from this highly conserved gene, with deposited sequences of this gene from other papillomaviruses, demonstrated a reasonably strong relationship (nucleic acid and amino acid identity) between this sequence and sequences from human and other animal PVs. Phylogenetic analysis recognised the proximity of the PPV to the human EV PVs and cutaneous PVs of other animals but demonstrated the uniqueness of the PPV by consistently placing the possum sequence as separate lineage.

A previously unknown cutaneous papillomavirus in a possum (*Trichosurus vulpecula*) was identified and determined to be a unique new type. This represents one of the first identified and reported viruses in this species.

Chapter 9

GENERAL DISCUSSION

Possums continue to be one of the major vertebrate pest species imported into New Zealand. Their impact threatens natural ecosystems through both direct and indirect effects on native flora and fauna in a diverse range of environments. While in areas modified by human endeavour possums damage a variety of farming, cropping and forestry operations by inappropriate browsing, the most significant economic problem attributed to possums is the maintenance of *Mycobacterium bovis* in a feral population that has direct contact with pastoral farming systems. Major costs to New Zealand accrue from ongoing expenditure to monitor and limit the prevalence and spread of bovine tuberculosis in endemic areas and to control possum populations by strategic application of poisons (notably 1080). Indirect costs have been incurred through research programmes aimed at understanding the impact of possums and possum control operations on native ecosystems, the mechanisms of *Mycobacterium bovis* transmission between possums and livestock (including transmission between susceptible feral species) and on research to develop improved and alternative methods of control. The research reported in this thesis was undertaken as part of the latter objective.

In addition to recording the viral flora of possums, the main aim of this project was to identify a possum specific virus that could be employed as a pathogen or as a vector for molecular biological modification and delivery of genes effecting immune mediated reproductive control (immuno-contraception).

The major achievements of the study were :

1. Development of two new cell lines from female possum reproductive tract tissues.
2. Confirmation that typical epiphytic warts on the tail of a possum contained papillomavirus DNA by PCR amplification of the L1 gene using redundant, consensus primers. Manual sequencing and subsequent phylogenetic analysis of 393 base pairs of the approximately 450 base pair product demonstrated a unique sequence and new papillomavirus type.
3. Demonstration that the agent of wobbly possum disease (WPD), a newly recognised neurological disease in captive possums, was of a size consistent with it being a virus. Histopathological features of the disease were described and a numerical case definition (weighted equally for clinical and histological features) was developed. Possible routes of transmission were investigated using a standard tissue suspension containing in excess of 10^5 PID₅₀ / ml. The tissue suspension (comprised of brain, liver and spleen) was infectious by the intra-peritoneal, intra-tracheal, intra-gastric and intra-dermal routes. Blood and urine from infected possums were shown to contain infectious virus. Individually caged and inoculated possums did not transmit disease to adjacent, non-contact controls but possums inoculated by the intra-peritoneal route passed the disease efficiently to possums which were in full contact with them. The main route of transmission in natural situations has yet to be determined but possum behaviour supports the possibility for both the oral route and the intra-dermal route to play a role.
4. Identification of a neurological disease of possums, clinically indistinguishable from WPD, in a population of wild possums near Rotorua. Histologically, this disease differed slightly from WPD in that the meningo-encephalitis was frequently mild even when possums exhibited a clinically severe neurological deficit. In contrast to WPD, possums inoculated with infectious material by the intra-peritoneal route did not invariably contract clinically recognisable disease. It is not known if the infectious possum disease from Rotorua is identical to WPD or a variant of WPD.

5. Screening of possum intestinal tract contents or faeces for virus particles by electron microscopy identified four samples containing adenovirus, presumed to be a possum-adapted strain. All four samples were subsequently shown to contain adenovirus DNA by PCR.
6. The survey of possums for viruses by tissue culture methodology was completed with modifications as described in chapter 3 but did not yield any viruses that were detected as replicating reliably in any of the tissue culture systems used.

It is worthwhile to consider the research reported in this thesis against the goals which existed when the research was proposed. None of the possum viruses identified to date (papillomavirus, adenovirus and WPD virus) could be considered as ideal candidates for modification towards biological control purposes, although a readily cultivable adenovirus would have considerable potential.

Adenoviruses, assuming the possum associated type is typical of the mammalian genus, do have a number of biological features that could be used to advantage in the design of a bio-control vector. Adenoviruses are non-enveloped and reasonably resistant in the environment, they utilise a faecal / oral mode of transmission and exhibit a high degree of host specificity (Fenner *et al.*, 1993a). Adenoviruses frequently cause mild or subclinical respiratory and enteric illnesses (although severe diseases are recorded) and the genome is large enough to manipulate and make genetic substitutions. For example, adenovirus vectors have been employed to express and provide immunity against the hepatitis B surface antigen (Morin *et al.*, 1987) and the stability of adenovirus-rabies recombinants in the environment has been assessed with large scale deployment in mind (Kalicharran *et al.*, 1992). Although research on the possum adenovirus has been slow to date due to technical difficulties with propagating the virus and the small amount of material with which to work, this candidate virus is worth more detailed investigation.

Papillomaviruses from any host species have never been cultivated using standard tissue culture techniques and the possum papillomavirus has yet to be propagated *in vivo*. Limited clinical material suggests that the way forward with this virus may be to clone the entire genome rather than risk a scant resource in further *in vivo* trials. The possum

papillomavirus also has potential for modification towards a biological control agent and shares with adenoviruses the properties of host specificity and resistance in the environment. Many research applications have demonstrated the usefulness of papillomaviruses as vectors for transfection of eukaryotic cells (DiMaio, 1987) and chromosomal integration of transcriptionally active foreign genes has been reported (Waldenström *et al.*, 1992). While the compact papillomavirus genome (~8kB) could potentially limit the size of a foreign DNA insert and direct the immunogenic strategy toward small yet crucial epitopes, the main problem identified with modification of a papillomavirus for biological control is the difficulty of *in vitro* manipulation. Techniques for *in vitro* cultivation of papillomaviruses are specialised (Meyers and Laimins, 1994; Kuo *et al.*, 1994; Mungal *et al.*, 1992; DiLorenzo *et al.*, 1992; Bossens *et al.*, 1992) and there are no reports of *in vitro* propagation of recombinant, infectious viral progeny.

Wobbly possum disease virus is insufficiently characterised for speculation on its potential for modification as a bio-control agent. Once WPD is classified, suitability for manipulation towards these ends can be extrapolated from the behaviour of other closely related viruses and strategic experiments performed. WPD differs significantly from the viruses thus far described in that it causes (experimentally) a severe fatal disease with a moderately prolonged clinical course. As a control agent in its own right, aspects of possum welfare would need to be carefully addressed against public and scientific concern for the 'quality' of the fate of a pest species (Gregory *et al.*, 1996; Loague, 1996). A second important requirement of candidate viruses for biological control is absolute host specificity. Wobbly possum disease must be shown to affect possums alone in order to gain acceptance for release as a modified organism. Other desirable properties of viruses with biological control potential such as ease of cultivation and laboratory manipulation, ability to be stably transfected and transmission and sustainability in the environment are of less importance than the welfare and safety issues outlined above. Wobbly possum disease has already been shown to be readily transmissible between possums in the laboratory but in this situation it causes a severe illness which may be unacceptable illness in welfare terms.

Although the survey using tissue culture techniques was expensive in terms of labour and

disposable resources and did not produce a tangible virus isolation, two new possum cell lines were developed and the techniques associated with growing possum primary cells *in vitro* refined. The lack of success in culturing WPD placed further demands on the development of alternative techniques for cultivation of possum cells and strategies for virus isolation. Tissue culture expertise with possum cells and more specialised procedures were important in the emerging field of possum virology where the adenovirus and WPD virus appeared to have fastidious cultural requirements (neither virus has been cultivated in possums cells).

A by-product of the survey work was the relatively high profile given to investigation of outbreaks of ill-health in possums in general. The possum papillomavirus was investigated in detail due to the availability of personnel interested in the astute observations of a field worker. The demonstration of a possible variant of WPD near Rotorua was made possible by the significant investment in protocols and methodology for investigating such outbreaks and the provision for storage of clinical material.

Advancing the knowledge base for each of the viruses identified should occur on parallel fronts due to the differing stages that research with each of the viruses has reached. Cloning of the possum papillomavirus and cultivation of the possum adenovirus are quite separate projects. Fortunately there are many sources of information that describe these processes for papillomaviruses and adenoviruses of other species. While several attempts to cultivate the possum adenovirus have not been successful, there are still many techniques to be trialled and work on the cloning of the possum papillomavirus has yet to be attempted. Investigation of WPD virus is at a more fundamental stage with characterisation, final identification and classification still to be completed. That WPD does not fit neatly into a defined virus group so far, makes work with this virus particularly challenging.

One of the most satisfying aspects of the research into possum viruses was the range of techniques that the various avenues of investigation opened up. Studies with WPD enabled the fundamental aspects of disease transmission to be considered and experiments designed at a gross level. At the opposite end of the spectrum, analytical

ultra-centrifugation for detection of WPD virus enabled concepts of solution gradient formation, macro-molecular complex and subcellular organelle movement under centrifugation to be studied. Immune-EM and agar gel immuno-diffusion (data not shown) developed the concept of virus (antigen) / immunoglobulin (antibody) interactions for diagnostic purposes and at a different macro-molecular level, the PCR and PCR-based sequencing techniques for investigation of the possum papillomavirus enabled an otherwise theoretical knowledge of molecular biological principles to be applied. Phylogenetic analysis of sequence data is based on complex mathematical and computational principles. Tuition and guidance with this aspect of the research was essential for a meaningful outcome. To be able to participate in building the foundation database for a newly discovered and seemingly novel possum virus (WPD) was a challenging and satisfying exercise (appreciated to some extent in hindsight).

It is inevitable that with the benefit of hindsight, weaknesses in the design and application of protocols will be identified and alternative approaches suggested. The following discussion is limited to the scientific protocols which could have been improved. The positive control experiment related to the survey using tissue culture techniques should have been undertaken earlier in the research period to validate the ability of the indicator cell lines to detect a virus in the presence of tissue extracts. An extension of this experiment could have produced a theoretical sensitivity for the tissue culture isolation system (number of infectious particles / ml detectable by indicator cells in the presence of various tissue extracts and concentrations). The possum kidney cell line produced at Wallaceville and the possum cell lines developed at Massey University may be broadly non-permissive. To investigate such a possibility, in the theoretically most sensitive of the marsupial cell lines for possum viruses, a very small number of mammalian viruses could have been used to challenge the survey indicator cells. The Glenfield isolate of the Macropod Herpesvirus (MaHV-1) did grow in the Massey ovary cells to low titre (10^3 - 10^4 TCID₅₀/ml) but (unusually) permissiveness appeared to remain low or decrease with passage (data not shown). No other viruses were inoculated onto the indicator cells.

Independently of the above suggestion, the use of primary possum cells as a main indicator cell line was identified as a laudable goal for all survey isolation work.

Although less convenient than cell lines in a high demand situation, it was early technical difficulties that led to the decision to use possum cells of low to moderate passage rather than primary cells. Once production of primary epithelial cells from possum kidneys had become reliable it would not have been too difficult to prepare primary cells routinely given the ready availability of possums in New Zealand. However, primary cells did not perform optimally in multiplates or chamber slides and other practical limitations were related to incubation and storage if small flasks were used for this approach or to the processing of multiplates containing glass inserts for haemadsorption and cytological examination.

Screening protocols to collect a larger pool of the possum adenovirus would have been worthwhile. Electron microscopy as a screening technique is relatively insensitive (10^6 particles / ml) and also very time consuming. A latex agglutination technique or other commercially available kit may have allowed many more faecal samples to be screened especially as the virus research project utilized only a small proportion of the possums collected by Landcare. The challenge to cultivate the possum adenovirus became increasingly appealing as skills and techniques were developed. In hindsight, considerably more effort into this aspect of the possum virus research could have been undertaken and this has subsequently been identified as a goal in its own right.

WPD transmission studies, although time consuming, produced a vast amount of clinical material and the challenge to produce meaningful data beyond the terms of the transmission experiments was undeniable (for example haematological investigations). The lack of a tissue culture isolate with which to work hampered other research efforts that required a source of the agent that was relatively free of tissue contaminants. Experimentation with WPD virus often took the form of an initial experiment to identify pitfalls followed by one or more subsequent attempts to gain useful data. In hindsight, perseverance with the techniques of preparative analytical ultra-centrifugation and more exhaustive attempts to purify the virus from the ample tissue stocks available could have provided a viral reagent / antigen of significant value in subsequent investigations. For example, the lack of suitably purified WPD antigen, specific anti-WPD antibodies and anti-possum antibody(s), from unrelated species, precluded the development of several

tests such as the ELISA and indirect immuno-fluorescent antibody test (IFAT). It had been suggested during the course of the survey that probing monolayers with pooled convalescent sera (IFAT) may have detected occult viral replication. Plans for the addition of immunological reagents to the armoury of possum virus detecting protocols were developed but not completed.

New Zealand is a small, geographically isolated country with a unique marsupial pest problem. The details of the survey methodology have limited appeal as they were developed with possum specific viral pathogens in mind. However, with no reports in the main stream literature of surveys undertaken to isolate viruses from a wild species in the absence of a disease syndrome, the development, testing, modification and fine tuning of the protocols reported here should be a valuable resource for other researchers contemplating a similar approach. Although a focused study, the research reported in this thesis adds to the knowledge on viruses of marsupials and significantly to the body of knowledge on viruses of possums, where there has not been a summary of recent literature and research findings.

The possum papillomavirus and possum adenovirus are likely to be fairly typical members of the genera they fall into. While research to record further details of these viruses is worthwhile to complete their characterisation, investigation of the epidemiology of these two potentially useful agents for biological control should have an equal priority.

WPD, on the other hand, appears to be anything but a virus typical of another well characterised family. Currently there is some serological and histopathological evidence linking this agent with a Borna disease (BD) like virus (O'Keefe, pers. comm.; Sabirovic, pers. comm.; Gosztonyi and Ludwig, 1995). Although no BD-like disease is known to exist in N.Z. domesticated animals, this is potentially a very interesting hypothesis as BD is gaining considerable attention world wide for its association with human neuropsychiatric illness (Lipkin *et al.*, 1995) and a number a BD-like illnesses have been described in other species, including cats (Lundgren *et al.*, 1995) and ostriches (Malkinson *et al.*, 1995). Whether or not the WPD agent is BD-like virus remains to be determined and there is evidence both for and against a BD-like aetiology at the present

time. Either way WPD should be investigated further. If BD-like, investigation of its niche in the possum population and its epidemiological profile will make an intriguing study in its own right but should preclude it from use as a biological control agent on the basis of an hypothesised lack of rigid host specificity. If WPD turns out to be a different virus or sufficiently different from BD that host specificity can be established, then it may be worthwhile investigating as a potential agent for biological control in spite of an apparently inhumane clinical course in its experimentally recognised form.

The possum problem in New Zealand has lead to a focused investment in basic virological research which has provided new skills in a specialised area and new tools for onward investigation of the findings. The discovery of a new viral disease of possums in both a research environment and a natural setting has demonstrated the dynamic nature of virus research. It is clear that from both a pure and an applied perspective there are a number of research avenues in possum virology requiring ongoing investigation. It is hoped that the progress made in recording and characterising possum viruses to date will encourage further research funding and that a similar investment will result in the isolation of a possum virus which is suitable as a pathogen or for modification and subsequent use as a biological control agent.

Appendix A1 - Tissue culture reagents.

ALSEVER'S SOLUTION

Sodium Citrate (Trisodium dihydrate)	8.0 g
Citric acid	0.55 g
NaCl	4.2 g
Glucose	20.5 g

Alsever's solution was made up to 1.0 litre with dH₂O, adjusted to pH 6.1 and sterilised by autoclaving at 10 lbs.

ANTIBIOTIC - TRYPSIN - VERSENE. (ATV)

Trypsin Difco, 1:250	0.5 g
Versene (EDTA) tetrasodium salt	0.2 g
NaCl	8.0 g
KCl	0.4 g
Dextrose	1.0 g
NaHCO ₃	0.58 g
Penicillin	2 x 10 ⁵ units.
Streptomycin	0.1 g
Phenol red	0.02 g

Trypsin solution for disaggregation of surface dependent cells was adjusted to pH 7.2, sterilised by filtration and 50 ml aliquots stored at -20 °C until required.

EDTA = Ethylene diamine tetra-acetic acid

BAYTRIL®

Baytril 2.5% (Bayer) equivalent to 25 mg Enrofloxacin / ml

Baytril was diluted 1:12 in PBS and used at a rate of 1% for transport medium or tissue culture (when required).

DISPASE®

Dispase (Gibco BRL)	1.0 g (lyophilised)
PBS	330 ml

PBS was warmed to dissolve dispase. Sterilisation was by filtration (0.22 µm). Antibiotics (Gentamycin or PSK) optional.

EAGLE MINIMUM ESSENTIAL MEDIA + minimum essential nutrients (MEM+n)

MEM (Sigma cell culture)	9.7 g
NaHCO ₃	2.2 g
Distilled H ₂ O	1.0 litre

Sterilisation was by filtration (0.22 µm). All filter sterilisation procedures were carried out in a laminar flow tissue culture cabinet using aseptic techniques. Aliquots of tissue culture media were incubated at 37 °C to test sterility.

FUNGIZONE®

Fungizone was supplied as a liquid containing 250 µg / ml (Gibco BRL).

Transport medium contained 1 % fungizone (2.5 µg / ml).

Culture media contained 0.1 % fungizone (0.25 µg / ml) when required.

GLUTAMAX™ - 1 Supplement (GIBCO BRL)

200 mM in 0.85 % NaCl (Supplied)

GROWTH MEDIUM (GM)

MEM+n was the basic growth medium to which was added

ELH	10%
TPB	10%
PSK	1% (fresh PSK if solution > 1 week old)
L-glutamine or	1% (fresh L-glutamine if solution > 1 week old)
GlutaMAX™	1% (fresh GlutaMAX™ if solution > 1 week old)
FBS	5-10% foetal bovine serum (Gibco BRL)

LACTALBUMIN HYDROLYSATE + Earle's salts (ELH)

ELH (Sigma cell culture)	13.7 g
NaHCO ₃	2.2 g
Distilled H ₂ O	1.0 litre (sterilise by filtration 0.22 µm)

L-GLUTAMINE (200 mM)

L-Glutamine	29.2 g
Distilled H ₂ O	1.0 litre (sterilise by filtration 0.22 µm)

MAINTENANCE MEDIUM (MM)

As for growth medium with a lower rate for the addition of FBS (1 - 2%).

PENICILLIN STREPTOMYCIN KANAMYCIN (PSK)

Streptomycin	1.0 g
Kanamycin	1.0 g
Penicillin	1 x 10 ⁶ units

PSK was made up in 100 ml of PBS, sterilised by filtration, dispensed into 20 ml aliquots and stored at -20 °C until required.

PHOSPHATE BUFFERED SALINE pH 7.2 (PBS) Ca^{++} and Mg^{++} free

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

PBS was made up to 1 litre with distilled water, adjusted to a pH of 7.2 and sterilized by autoclaving.

PRIMARY MEDIUM (PM)

As for growth media with a higher rate for the addition of FBS (15- 20%) and between 10 and 50% of the growth medium substituted for filtered conditioned media (FCM) To produce FCM, medium was preferentially recovered from healthy primary or low passage possum cells following 3 - 7 days, centrifuged and filtered (0.22 μm).

TISSUE CULTURE MATERIALS

Nunclo [®]	50 ml, 260 ml and 800 ml polystyrene tissue culture flasks.
Nunclo [®]	96 well and 6 well multiplates.
Falcon [®]	24 well, flat bottomed multiplates.
Nunclo [®]	8 well chamber slides.

TRANSPORT MEDIUM (TM)

Transport medium consisted of PBS containing 1% PSK and 1% fungizone (Gibco, BRL) for samples that were collected aseptically. Transport medium for samples that were contaminated at origin (for example tonsils) or difficult to collect using a sterile technique (for example ganglia) consisted of PBS containing 2% PSK, 1% Fungizone and 1% Baytril.

TRYPTOSE PHOSPHATE BROTH (TPB)

Tryptose	20.0 g
Dextrose	2.0 g
NaCl	5.0 g
Na_2HPO_4 (anhydrous)	2.5 g

TPB was made up to 1 litre with distilled water, adjusted to pH 7.3, dispensed into 200 ml aliquots and sterilised by autoclaving at 10 lb for 15 minutes.

Appendix A2 - Molecular biology reagents.

AMMONIUM ACETATE (5 M)

CH ₃ COONH ₄	3.85 g
Distilled H ₂ O	10.0 ml (sterilised by filtration)

K BUFFER pH 8.3

PCR buffer (pH 8.3) (Boehringer-Mannheim)	10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl ₂
Tween 20	0.5 % (v/v)
Proteinase K	100 µg/ml

PROTEINASE K Stock solution (10 µg/µl) in TE buffer

Proteinase K	2.6 mg
Distilled H ₂ O	260.0 µl

TAE (Tris/acetate/EDTA) electrophoresis buffer pH 8.5 : 50 x stock solution

Tris-Cl base	242 g
Glacial acetic acid	57.1 ml
EDTA.2H ₂ O (di-sodium salt)	37.2 g
Distilled H ₂ O	1.0 litre

Working solution (pH 8.5)	40 mM Tris-acetate 2 mM EDTA
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TBE (Tris/borate/EDTA) electrophoresis buffer pH 8.0 : 10 x stock solution

Tris-Cl base	108 g (890 mM)
Boric acid	55 g (890 mM)
EDTA (0.5M) pH 8.0	40 ml (20 mM)
Distilled H ₂ O	1.0 litre

Working solution (pH 8.0)	89 mM Tris 89 mM Borate 2 mM EDTA
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TE BUFFER (10 mM Tris-Cl, 1 mM EDTA) pH 7.5 - 8.0

Tris stock (1 M)	12.11 g	Tris-Cl base
Distilled H ₂ O	80.0 ml	adjust to pH 8.0 (conc. HCl) make up to 100 ml
EDTA stock (10 mM)	0.372 g	EDTA (di-sodium salt)
Distilled H ₂ O	100 ml	

TE buffer 200 µl Tris stock (1 M) pH 8 and 2 ml EDTA stock (10 mM).
Add 16 ml distilled H₂O (16 ml) and adjust to pH 7.5. Make up to a
total volume of 20 ml with distilled H₂O.

Appendix A3 - Electron microscopy reagents.

CAESIUM CHLORIDE STOCKS

CsCl (anhydrous)	26.33 g
Distilled H ₂ O	100 ml
Density of solution (1.1989 g/cm ³)	
CsCl (anhydrous)	52.96 g
Distilled H ₂ O	100 ml
Density of solution (1.3989 g/cm ³)	
Method of gradient preparation	Chapter 5

GTNE BUFFER (pH 7.5)

Glycine	200 mM
Tris-Cl	50 mM
NaCl	100 mM
EDTA	1 mM

Method: Stock solutions were prepared at 10 x the working molarity. Appropriate volumes to produce the working molarity of each ingredient were added to 800 ml of distilled H₂O. The pH was adjusted to 7.5 and the final volume adjusted to one litre.

LEAD CITRATE

Pb citrate	1.5 g
Pb acetate	1.5 g
Pb nitrate	1.5 g
Distilled H ₂ O	90 ml

Method: Heat ingredients to 40 °C (stirring), hold for one minute, add 3 g Na citrate and stir further for a further minute. Add 24 ml 0.1 M NaOH (fresh) and 24 ml dH₂O.

PHOSPHATE BUFFER (pH 7.2 - 7.4)

Na ₂ HPO ₄ (0.2 M)	180 ml
NaH ₂ PO ₄ 2H ₂ O (0.2 M)	70 ml
Distilled H ₂ O	500 ml total volume
Working solution molarity	0.1 M

PHOSPHATE BUFFER STOCK SOLUTIONS (0.2 M)

Na ₂ HPO ₄ (0.2 M)	14.2 g 500 ml distilled H ₂ O
NaH ₂ PO ₄ 2H ₂ O (0.2 M)	7.8 g 250 ml distilled H ₂ O

PHOSPHOTUNGSTIC ACID STAIN

Potassium phosphotungstate	2 g
Distilled H ₂ O	100 ml

Method: Dissolve potassium phosphotungstate, adjust to pH 7.2 with KOH and filter (0.22 μ m).

SUCROSE STOCK SOLUTION

Sucrose 66% w/w	171 g sucrose
GTNE	99 ml

SUCROSE GRADIENT STOCKS

Sucrose 20%	24.5 ml sucrose stock (66%)
GTNE	75.5 ml
Sucrose 55%	80.5 ml sucrose stock (66%)
GTNE	19.5 ml

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ERRATA

PAGE / LINE NO.	ALTERATION TO TEXT IN BOLD FONT
viii / 10	4.3.9 Inoculation of possums with rabbit tissues.....109
x / 13	Table 4.8 Haematological data for eight possums before and after inoculation with WPD.
2 / 13	(Brockie and Moeed, 1986)
2 / 27	(Seitzer <i>et al.</i> , 1992)
12 / 15	Presidente's summary
16 / 6, 22	(Acland , 1981)
37 / 3	(Sasaki and Ludwig , 1993)
42 / 4	reported (Gross , 1970).
79 / 27	(Spector and Lancz , 1986)
111 / 16	(Blood et al. , 1983)
160 / 8	(Day et al. , 1996)
241 / 39	Additional reference : Bowen, G.S. 1976. Experimental infection of North American mammals with epidemic Venezuelan encephalitis virus. <i>American Journal of Tropical Medicine and Hygiene.</i> 25, no. 6: 891-99.
249 / 16	163. Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley, and S.T. Williams (editors). 1994.
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