Towards the Creation of a Transgenic Possum Parasite

by

Jan Newton-Howes

A thesis submitted to
the Victoria University of Wellington
in fulfillment of the requirements for the degree
of Doctor of Philosophy
in Molecular Biology and Biochemistry

Victoria University of Wellington
2002

Abstract

The brushtail possum, *Trichosurus vulpecula*, is New Zealand's most serious vertebrate pest; possums destroy native flora and fauna and are vectors of bovine Tb. Conventional control is considered to be unsustainable and, in the long term, biological control is seen as the only solution to reducing possum numbers.

The aim of this project is to contribute to the development of a self-disseminating vector that will spread a control molecule throughout the possum population reducing fecundity or increasing mortality. The possum-specific parasite *Parastrongyloides trichosuri* has considerable potential as such a vector.

A protein from *P. trichosuri* specifically, was found to be antigenic in possums. The antibodies to this protein were purified from positive possum serum and used to detect the antigen on the surface of infective larvae but not in the excretory/secretory products of either larvae or adults. The protein was isolated from crude infective larvae and found to show homology to the heat-shock 70 family of proteins.

Genomic DNA was extracted, an oligonucleotide probe made and a genomic library screened for the Hsp70 gene. Several positive clones were found and DNA isolated and sequenced from one such clone. Five kilo bases of unambiguous sequence was obtained in which was an open reading frame of 2 kb. Theoretical translation of this gave a protein of 64 amino acids with 80% homology to the Hsp70A protein of *C. elegans*. The region upstream of the ATG initiator codon was amplified and 1.3 kb of the putative promoter region was cloned into a vector containing the *gfp:lacZ* reporter genes.

This construct was microinjected, first into *C. elegans* to demonstrate promoter function, and then into both free-living and parasitic adults of *P. trichosuri*. Reporter gene expression was shown in the progeny of microinjected parasitic adults.

RNA was made from infective *P. trichosuri* larvae, reverse transcribed and the coding sequence for the PtHsp70 protein cloned into an expression vector and expressed in *E. coli*. The recombinant protein pattern had a similar pattern of trypsin digestion products as the native protein, as shown by MALDI-TOF mass spectrometry, but it was immunologically distinct from the native protein.

The culmination of this project was the generation of a transgenic *P. trichosuri*, the first vertebrate endoparasitic nematode to be heritably transformed. This is a necessary step in the development of a self-disseminating vector to be used in the biocontrol of possums.

Acknowledgements.

I should like to acknowledge my supervisors at AgResearch, Wallaceville where all the experimental work for this project was carried out. Firstly, David Heath who was responsible for the idea for this work, who was involved in all the early stages and who accepted me in the first place in a most unprejudiced manner. Secondly Chuck Shoemaker for all his expert help and involvement during the molecular biology stages despite an increasingly busy schedule. I should like to thank them especially for their support during the interruptions to this work, which was greatly appreciated.

At Victoria University, I should like to thank John Andrews who guided me into the project and continued to monitor and encourage me, and, latterly, Bill Jordan for agreeing to shepherd the thesis through the final examination steps.

At AgResearch there are many people who willingly shared their time and expertise to help me learn the many techniques I needed. Among them I should like to thank: Alan Gruenberg, Pam Kawakami, Jacqui Knight, Dave Maas, Margaret McNeil, Chris Robinson, Richard Shaw, Susan Stasiuk. I should also like to thank Warwick Grant for his helpful discussion of this thesis.

I must thank, in particular, the other "possum people", Mark Ralston and Steve Skinner. To Mark I gladly give the "D" of this PhD in return for all those possums, and without Steve's tender loving worm-care there might never have been a green worm.

Thanks also to Ruth Kiel for drawing the diagrams and help with other illustrations, to Michelle Nesbitt for typing all the tables, and to Derek Kane for IT support above and beyond the call of duty.

I was supported by a scholarship from AGMARDT, New Zealand, and the work itself was funded by grants from MAF through the NSSC.

I also want to acknowledge the great support and encouragement I have had from my friends and family, especially my husband Gordon Smith who has never been anything other than totally supportive and has never once cursed my work, even when I have. Thank you all.

Contents

II Identification of the immunogenic protein

III Cloning and Analysis of a *P. trichosuri* Hsp70 Gene

IV Production of *P. trichosuri* Hsp70 promoter construct and microinjection experiments

List of Figures:

List of Tables:

Chapter I Introduction

The common brushtail possum *Trichosurus vulpecula* is one of New Zealand's most serious mammalian pests. It destroys large areas of native bush and harms native fauna directly and indirectly by habitat destruction and competition. It is a vector of bovine tuberculosis and as such has the potential to cripple New Zealand's agricultural exports causing severe disruption to the country's economy. Research on all aspects of the possum has accelerated since the 1970s as the absolute number of possums has increased and they have become implicated in the spread and maintenance of bovine tuberculosis. Their anatomy, immunology and physiology, with particular emphasis on their reproduction, have been investigated and their behaviour, ecology and effect on the environment have become more comprehensively documented. This literature is now reasonably widely available and has been effectively collated but, as the problem of the possum in New Zealand is the fundamental issue from which this project derives, Sections one to three of the introduction will briefly review the above topics.

1.1] The Common Brushtail Possum, *Trichosurus vulpecula*

1.1.1 The Possum- classification

Trichosurus vulpecula [Kerr]

Class: Mammalia

Order: Marsupialia

Sub order: Diprodonta

Family: Phalangeridae

Genus: *Trichosurus* - hairy tail

species: *vulpecula* - little fox

The common brushtail possum or opossum, *Trichosurus vulpecula* [Kerr 1792] is a native Australian marsupial. The origin of marsupials remains uncertain, however the separation of marsupial from eutherian mammals is currently thought to have occurred between 71-170 million years ago [mya] [Hope, 1990]. The earliest marsupial fossils are from the Cretaceous period in North America and currently North or South America

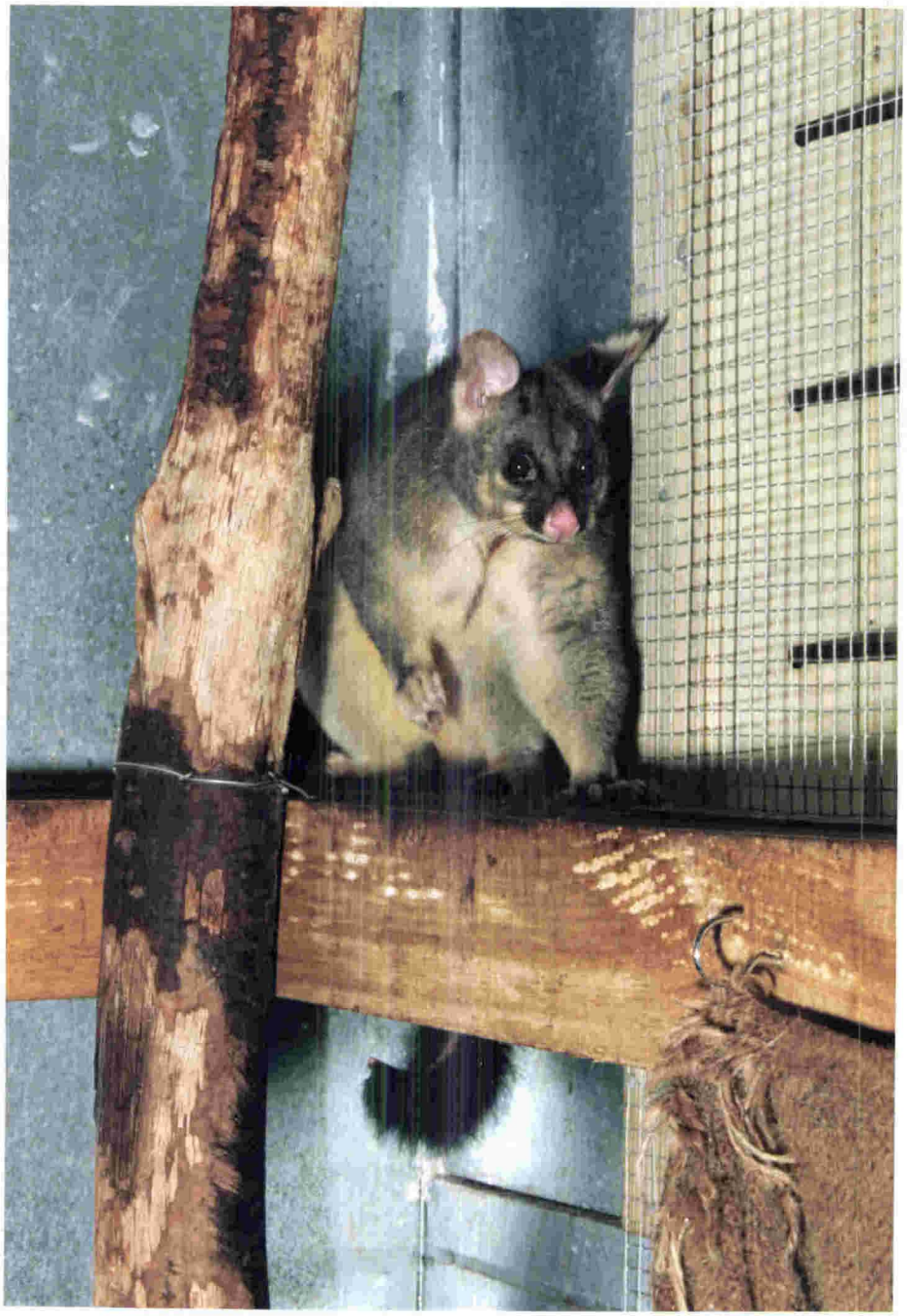


Fig 1.1 The brushtail possum, *Trichosurus vulpecula*, housed at Wallaceville Animal Research Centre

is favoured as the geographical region of origin. Dispersal to Australia probably occurred in the late Cretaceous, 75 mya, at which time Australia, Antarctica and South America formed a single land mass. Any eutherian mammals present in early Australia disappeared and marsupials remained isolated until about 10 mya with the collision of Australia/New Guinea with the Indonesian archipelago. Despite uncertainties in dates, there has been a period of at least 50 million years during which the Australian marsupials have existed in isolation and they have diversified into all major habitats.

Trichosurus vulpecula belongs to the marsupial order Diprodonta, that is, it differs from other marsupials in having a single procumbent pair of lower incisors. The family Phalangeridae comprises 10 species of *Phalanger*, the cuscuses, one species of *Wyulda,* [scaly tailed possum], and three species of *Trichosurus.* The genus *Trichosurus* or brushtailed possums comprises three species: *caninus*, the mountain possum living in rainforests in southern Queensland and NSW and parts of Victoria, *arnhemensis* found in Arnhemland and northern parts of the Northern Territory, and *vulpecula* which is widely distributed throughout other wooded areas [Smith and Hume, 1984]. The grey and the larger black form of the species *T. vulpecula* were introduced into New Zealand.

A full, detailed description of the anatomy, reproduction and development, social organisation and feeding behaviour of the possum is given by Cowan [1990]. The possum, shown in Fig 1.1 is a thickly furred, grey or reddish black animal with a fox-like pointed snout and large ears. It is about the size of a cat, on average 0.8m long with a thick furry prehensile tail of about 0.3m; the adults weigh between 1.4 - 6.4 kg.

1.1.2 Reproduction.

Females become mature and breed at between one and two years in an established population; in a colonizing population as many as half the one-year-old females breed [Green, 1984]; the males become mature between one and two years of age. Breeding in most populations so far studied is highly seasonal, mid April to mid June being the peak breeding months, although some births have been recorded in every month of the year [Kerle, 1984]. Where females are in good condition and autumn has been early, it is possible for some individuals to breed again in spring. The occurrence of double breeding appears to correlate with latitude in Australia, [with possums living at lower

latitudes having a higher incidence of spring births] as well as food supply and consequent condition of females [Humphreys *et al.*, 1984] and it also takes place in colonizing populations [Green and Coleman, 1984].

The reproductive physiology of both male and female possums has recently been extensively studied. In males there is an annual cycle of secretion of testosterone that results in increasing prostate size to a peak at the beginning of the breeding season in March [Gemmel *et al.*, 1986]. Spermatogenesis appears to take place in similar stages to eutherian mammals but, in contrast to eutherians, as the sperm leaves the testis and moves through the epididymis the acrosome undergoes marked change in shape from an elongated scoop to a compact button shape [Lin *et al.*, 1999]. Sperm transport and capacitation also show some possum- or possibly marsupial-specific features; within the female tract, usually the oviduct the sperm head changes orientation to a T or thumbtack morphology with the head at 90° to the flagellum possibly due to a sudden decrease in calcium ion concentration [Rodgers *et al.*, 2001, Molinia *et al.*, 2001]. This change indicates capacitation and sperm with this shape can be seen binding to and penetrating the zona pellucida of the egg.

The female possum undergoes oestrus cycles of 26 days until conception occurs. One offspring only is conceived normally and gestation is very short, the young being born after 17 -18 days gestation when the minute, immature animal weighing about 0.2g climbs through the fur from the urogenital opening to the pouch. It then attaches to the teat and remains attached for 70 days. Development in utero and in the pouch is well documented [Fletcher and Selwood, 2000].

In contrast with gestation, lactation in marsupials is long, approximately 200 days in the possum. There are three distinct phases: the first involves the development of the mammary gland in preparation for lactation in the days just prior to and following birth of the pouch young. The second phase is marsupial specific and differs from early lactation in eutherians; milk production and prolactin levels are low and this milk comprises carbohydrate and protein with very little fat [Cowan, 1989]. It does, however, supply all the requirements for the continuing development and initial growth of the young. There are a number of unique proteins in this milk whose levels rise then

suddenly fall at the crossover or switch time to the third phase of lactation at around 120 days after birth [Demmer *et al.*, 1998]. During the third phase milk volume and fat levels increase with a change in protein composition occurring; the pouch young suckles intermittently. Composition is more similar to that of eutherians and milk production and growth of the young is high during this phase.

The young emerge from the pouch for increasing periods from about 120 days, first as back young then as fully independent young at about 240 -270 days. At independence most young females remain in their mother's home range and establish themselves there whereas young males disperse away from their natal area.

1.1.3 Diet and Feeding Patterns.

Possums have been described as "generalists and opportunistic folivores", leaves make up the bulk of their diet but they also eat shrubs and grasses, flowers, fruit and invertebrates [Cowan, 1990]. They have been caught on camera eating eggs and chicks of New Zealand native birds [Brown *et al.*, 1993] and will eat cooked or raw meat when offered it [Nugent *et al.*, 2000]. In Australia possums chiefly eat the foliage of eucalyptus species but have also adapted to a range of introduced shrubs and trees. In New Zealand at least 70 indigenous and introduced trees, 20 fern species and shrubs and grasses are eaten [Kean and Pracy, 1953 in Cowan, 1990]. With the ability to consume such a wide range of plants possums have been able to spread into almost every terrestrial habitat in New Zealand. In different habitats their diet consists of whatever particular species are available but in all cases the bulk of what is consumed derives from one or two of the species present; consequently these are intensively browsed. It is this feeding behaviour which puts great pressure on just a few species and that tends to increase their detrimental impact on native ecosystems. It may lead to either catastrophic dieback for instance, the rata/kamahi forests of the West Coast [Rose *et al.*, 1992] or severe compositional change composition in some forests as preferred species are browsed out even to extinction e.g. fuchsia [*Fuchsia excorticate*], rata [*Metrosideros robusta* and *umbellata*] and kamahi [*Weinmannia racemosa*] [Green 1984, Nugent *et al.*, 2000]. Studies of possum diet including the species consumed are summarized in Nugent *et al.*, [2000].

Even within a single species possums appear to favour particular individual trees that may be eaten to death before adjacent members of the same species are browsed. This may be a continuation of feeding patterns developed in Australia, postulated to be in response to the high levels of toxic secondary products, terpenes tannins and other phenolics, in the leaves of eucalypt species [Freeland and Winter, 1975]. More recent evidence has shown, however, that species that normally encounter terpenes in their diet have developed efficient oxidation pathways to eliminate them [Boyle *et al.*, 1999]. This difference in palatability among individuals of the same species has been investigated using a "bioassay-guided fractionation protocol" in which specific fractions extracted from browse-resistant and browse-susceptible *Eucalyptus ovata* trees were added to the diet of captive ring-tailed opossums. A specific terpene-type molecule called macrocarpal G was shown to be a highly significant feeding deterrent in this species [Pass *et al.*, 1998]. Levels of specific phenolic compounds, diformylphloroglucinols have been shown to affect dietary preferences in other marsupials and mammals [Lawler *et al.*, 1998] and may also play a part in possums' choice of tree in New Zealand despite the fact that this group of compounds is not present in New Zealand native flora to similar levels as in eucalypts.

From Australian data it can be seen that possums are able to survive on foliage alone [Fitzgerald, 1984] but it is thought that this is a subsistence diet only and flowers, fruit and soft-leaved or nitrogen rich species of herbage are always eaten in addition when available in New Zealand. Nugent *et al.*, [2000] postulates that the levels of these foods, which are both higher in nitrogen and energy and are more easily digested, are the limiting factor for possum density, and influence the carrying capacity of an area, rather than the availability of the bulk foliage component of their diet.

1.1.4 Activity and Behaviour.

Possums are solitary, nocturnal animals retreating to dens in hollow logs etc during the day. They are usually active from about thirty minutes after sunset to shortly before dawn in summer but when cold or wet they retreat early after eating. They have a bimodal activity pattern for feeding spending on average 1-2 hr per night. Social activity is unimportant except for mating. Possums occupy home ranges of from 1-7 hectares, size depending on the type of habitat; males have larger home ranges than

females and once established these tend to remain stable for both sexes. Home ranges can overlap, as possums in New Zealand are not usually territorial but do have dominance hierarchies dependent on size and age [Cowan and Clout, 2000]. Young males disperse away towards the end of their first year when becoming sexually mature; this dispersal appears to be independent of population density [Cowan *et al.*, 1997]. Young females usually establish home ranges close to their mothers.

When possums are eliminated from an area the population tends to recover initially through the dispersal of juveniles, mainly but not exclusively males, then in subsequent years by breeding of survivors and of immigrants. Possums that are established in areas contiguous with the controlled area do not generally take advantage of the lowered densities, vacant den sites etc, to move in [Green and Coleman, 1984], so the idea that control operations have a kind of vacuum effect does not seem to be confirmed [Cowan and Clout, 2000].

1.1.5 Immunology

Anatomy of the lymphatic system of *T. vulpecula* has been described [Jackson and Morris, 1996], as has the organized lymphoid tissue of the mucosal immune system of the gut [Hemsley *et al.*, 1996]. Although the majority of the structures of the system appear to be similar to those of other marsupials studied there are differences in organization from the typical system of eutherians, e.g. paired thoracic ducts, a thoracic and a cervical thymus, this latter is predominant. The pouch young are immunologically immature at birth, protection is via maternal immunoglobulins secreted into the milk, but by 25 days the thymus has differentiated and CD3+ T cells and IgG+ B cells are present [Old and Deane, 2000].

Four of the five immunoglobulin classes found in eutherian mammals have been detected in *T. vulpecula*. IgM and IgG have been isolated from serum [Ramadass and Moriarty, 1992] and IgA protein has been isolated and purified from possum milk [Doolin *et al.*, 2001]. Recently a number of possum immunoglobulin genes have been cloned and sequenced: the constant region of the heavy chain for IgM, IgG, IgE and IgA [Cμ, Cγ, Cε, Cα]. A single Cγ sequence only has been found in the possum despite evidence of multiple IgG subclasses [Miller and Belov, 2000]. The heavy chain variable

regions for IgA has been cloned [Adamski and Demmer, 1999] but not for other isotypes and the κ light chain has been cloned also [Belov *et al.*, 2001]. The constant region of the T cell receptor, TCRα and β chains have been sequenced and shown to have a high level of homology with the eutherian [human] receptor [Zuccolotto *et al.*, 2000].

The genes for some cytokines have been found: TNFα [Wedlock *et al.*, 1996], IL-1β [Wedlock *et al.*, 1998], IL-10 [Wedlock *et al.*, 1999], IL-5 and TGFβ [Wedlock unpublished]. These have been compared with their eutherian homologues and are relatively conserved, about 63% at the nucleotide level. This is considered surprising because of the rapidly evolving nature of cytokine genes but this may be due to their detection depending on homology. It has generally been assumed that marsupial immune responses will differ only marginally from eutherian but there is evidence of differences emerging, e.g. in the class switching of antibodies and cell mediated responses to secondary skin grafts [Harrison and Wedlock, 2000].

Mucosal immunity in the possum is even less explored but mucosal responses to a model antigen [KLH] have been stimulated and detected in serum and female reproductive tract secretions in response to intranasal immunization [Doolin *et al.*, in press]. Further work on the development and regulation of immune responses, both systemic and mucosal, are underway as these have significance for the possum biocontrol programme.

1.2] Introduction and Effect of the Possum in New Zealand.

1.2.1 History of the introduction and liberation of the possum in New Zealand

A complete account of the introduction and liberation of the possum is given by L T Pracy [1974]. Possums were introduced into NZ deliberately to establish a fur trading industry, with the earliest known successful release taking place in the Riverton area prior to 1840. Original release sites of imported possums are shown in Fig 1.2. Perhaps the most striking piece of information is that the total number of possums recorded as having been imported is between 200 and 300 possums [Pracy, 1974] whereas the

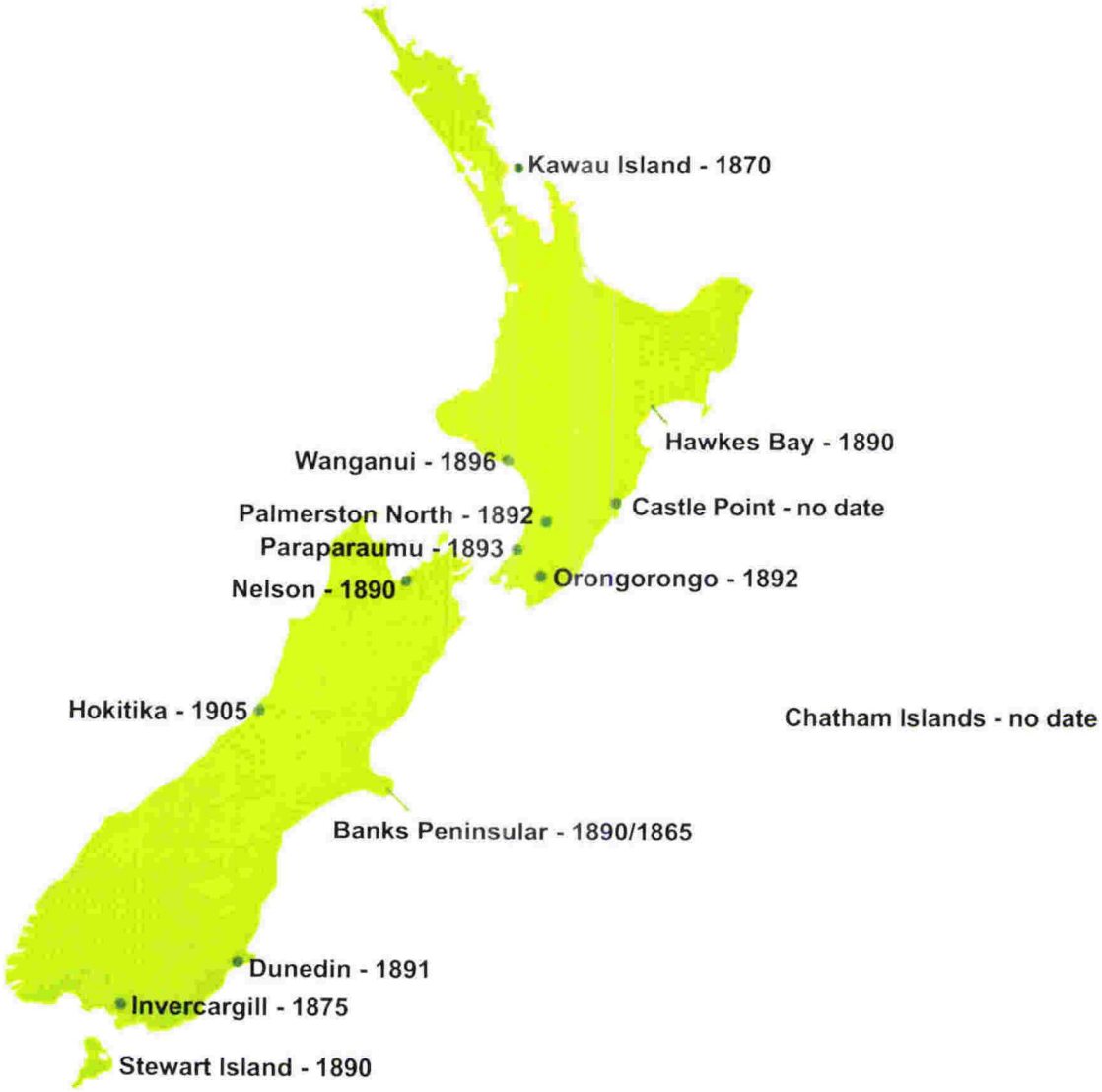


Fig 1.2 Places and dates of the original releases of imported possums into New Zealand in the 19th century

possum population of New Zealand one hundred and fifty years later has been estimated at 70 million [Batcheler and Cowan, 1988]. This is likely to be reasonably accurate as importations, as distinct from local liberations, were probably carefully recorded.

Liberations of New Zealand born possums by acclimatisation societies, government departments and private individuals were considerable; one recorded liberation, of two black possums into the grounds of Government House, took place at the request of the Cabinet in 1898. By 1922 the Department of Internal Affairs was declining all requests for permission to liberate possums because of the mounting evidence that they were destructive of both horticulture and natural forest but illegal liberations continued until the 1940s. In 1947 all regulations on killing possums were removed and poisoning in certain areas was legalized [Pracy, 1974]. The status of the possum has therefore vacillated between valuable game resource that was protected by the government who received royalties etc to a pest that damaged crops and plantations and could therefore be destroyed without control.

From the original sites of liberation, possums have progressively colonized all parts of New Zealand with the possible exception of some areas of southwest Fiordland. Possums were recorded in all parts of Northland, one of the last possum-free areas, in 1990. This has occurred despite possum control operations aimed at preventing their further spread. However, eradication of all possums from a number of islands has been completed successfully in the last decade with significant beneficial effects on native flora and fauna [Clout and Ericksen 2000].

1.2.2 Differences in possum populations between Australia and New Zealand.

In Australia possums are the most widely distributed marsupial, they are present throughout the mainland, Tasmania and on some offshore islands, occupying many different types of habitat [Strahan, 1983]. Population densities in most of their native habitats are usually below 1 per hectare rising to a maximum of 4/ha in Tasmania [Kerle, 1984]. In New Zealand they have been successful invaders and have adapted to a wide range of habitats in their new country. Here possums have attained large populations at far higher densities, up to twenty times higher, than in their native

Australia. Densities range from 1/ha in beech forests in the South Island to over 25/ha in podocarp broadleaf forest [Efford, 2000], see Fig 1.3.

Population size and density follows a pattern as possums colonize new areas; there is a slow but steady increase in numbers followed by an explosive increase to a peak of population which overshoots the carrying capacity of the environment. This results in a sharp decline in numbers. Long-term population studies have been carried out in the Orongorongo valley [Allen *et al.*, 1997] and at Pararaki, southern Wairarapa, [Coleman *et al.*, 1999]. The latter study supports an irruptive fluctuation model for possums and their food supply but it is not clear that this regulates population densities in other areas [Efford, 2000].

These enormous differences in population densities are due to a number of factors:

1) Diet. In Australia this is largely eucalyptus leaves with their lower level of nutrition and higher level of unpalatable phenolic compounds compared with the soft-leaved higher nitrogen foliage plus other high-energy food that is consumed in New Zealand.

2) Habitat. *T. vulpecula* are found chiefly in open eucalypt forests and seldom in wet sclerophyll and rain forest in Australia despite this being the habitat in New Zealand where they are found in highest numbers. It seems likely that *T. vulpecula* is out competed by *T. caninus*, the mountain possum in these favoured areas [Kerle, 1984]. There is competition from the many other species of possums and gliders for food and, particularly, den sites in all Australian habitats that is absent in New Zealand.

3) Predators. There are no native predators of possums in New Zealand though some, the young in particular, are preyed on by feral cats in the bush. Possums here are killed in greatest numbers by hunters and by cars on roads. In Australia they have more predators: dingoes, feral dogs and cats, birds of prey and some snakes and, importantly, introduced foxes [Cowan, 1990]. A study in Western Australia showed a fourfold increase in possum numbers after foxes were successfully controlled [Anon, 1997 cited in Cowan *et al.*, 2000 p82]. The effect

4)

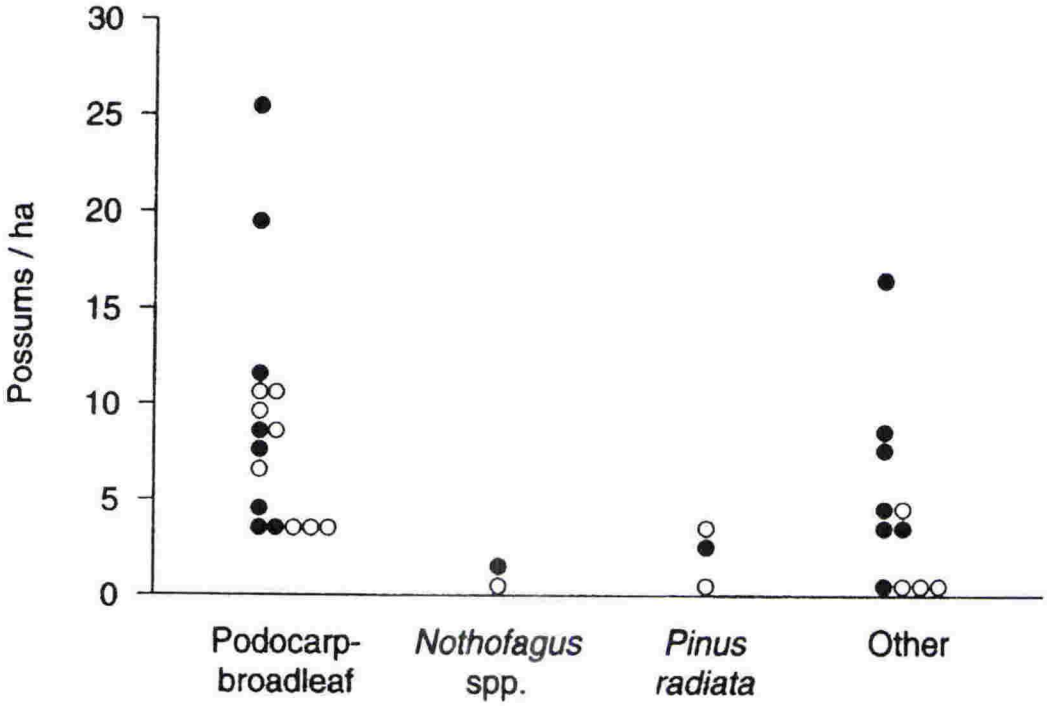


Fig 1.3 Possum population density, as possums per hectare, in different types of New Zealand habitat. Each symbol represents the average density from an intensive study of an undisturbed population. Solid circles indicate densities on pasture/forest margins, open circles indicate forest studies.
From Efford 2000, p48 (reproduced with permission)

of predation on herbivorous species such as possums is often discounted with the regulation of population numbers being attributed to limitation of food supply, particularly the low quality of forage and chemical defences in plants such as eucalypts. But recently the "top-down" hypothesis has been bolstered by the finding of huge increases in population densities of herbivores, from ten to one hundred times in areas [islands] from which predators have disappeared compared with control areas where predators remain [Terborgh *et al.*, 2001].

5) Pathogens and parasites. Possums in New Zealand have far fewer pathogens and parasites than those in Australia. Whether this is due to a favourable [to possums] founder effect or whether some of these organisms have been lost from the population due to unfavourable climatic conditions or lack of a suitable intermediate host [e.g. suitable mosquito species for the Filarioidea] is in most cases uncertain. Parasites are discussed in Section 4.

1.2.3 Effect on New Zealand native flora and fauna.

The high density of the possum population plus the preference-based nature of their eating habits has had a marked effect on native flora. It has been apparent from the 1950s that possums are implicated in forest canopy dieback and the changing composition of native forests [Chavasse, cited in Payton, 2000 p112]. There has been controversy over the extent to which the damage can be attributed to possums alone and other mammalian pest species such as goat and deer are also incriminated. Natural climatic factors such as drought and earthquakes almost certainly play a part in the multi-causal damage to native forests [Payton, 2000].

As well as the direct effect of browsing, possums are implicated in the inhibition of regeneration. Although ungulates are thought to be the main cause of this due to their eating of seedlings, possums' high consumption of fruit may have an effect on the regeneration of these species even if the foliage is not a bulk item of diet [Allen and Rose, 1983, Nugent, 1995]. Effects of possums on the native forests throughout the country are reviewed in Payton [2000].

The effects on native fauna are both direct and indirect. Possums are seen as competing with native birds for seasonal resources, fruit especially, e.g. there is significant overlap between the diet of the North Island kokako [*Calabash cinera*] and the possum [Fitzgerald 1984a], and nests sites with, for instance, kiwi [*Apteryx* spp.]. Recently there has been direct evidence of possums killing kokako eggs and chicks. Using time a lapse video of kokako nests, Brown *et al.*, showed that killing by possums caused the failure of four of the 19 nests filmed [Brown *et al.*, 1993]. There is strong evidence that possums have killed eggs, chicks or adults of at least six native bird species [Innes, 1994]. Faecal analysis has shown that large numbers of invertebrates are consumed [Cowan and Moeed, 1987]; cicadas, wetas, beetles, stick insects and the endangered native snail of the genus *Powelliphanta* have become prey species [Innes, 1994].

1.2.4 Possums as reservoirs and vectors of bovine Tb.

In 1967 the first possums infected with bovine Tb, *Mycobacterium bovis* were found [Ekdahl *et al.*, 1970]. Shortly after in the early 70s the failure to eradicate bovine Tb from certain herds of cattle was linked to the presence of possums infected with tuberculosis on the pasture/forest margin [Stockdale cited in Coleman and Livingston, 2000 p223]. Possums are highly susceptible to *M. bovis*; they develop widespread macro and micro lesions in many areas of their body. Survival time, post-infection, is thought to be about six months but may be as long as three years in the wild; transmission between possums can be from mother to young, through fighting or mating or contamination of shared environments especially den sharing [Coleman and Caley, 2000]. Tb persists in possum populations without nearby cattle or other obvious animal source, evidence that they are a wildlife reservoir for the disease [Pfeiffer cited in Coleman and Caley, 2000 p101].

Tuberculous possums have been shown to shed Tb organisms into the environment [Jackson *et al.*, 1995] and it is thought that possums infect cattle when sick or dying possums move out into pasture and are nosed by inquisitive stock, behaviour that has been observed [Paterson and Morris, 1995]. Cattle probably become infected via the respiratory route rather than through eating contaminated pasture but the route of

infection from cattle to possums is not known. Other wildlife can also become infected with *M. bovis:* deer, goats, cats, pigs and ferrets and stoats. These species are not thought to be reservoirs or vectors but dead end hosts with the possible exception of ferrets. It has been shown that it is more likely that possums infect ferrets than vice versa [Caley, 1998]; likewise possums probably infect deer [Lugton *et al.,* 1998].

The incidence of bovine Tb in New Zealand's beef and dairy herds is of extreme importance for its agricultural exports and therefore its economic well-being. Approximately 97% of the herds are officially free of Tb but the threshold of 99.8% must be reached for the country to be considered Tb-free. Some of our trading partners and competitors have this Tb-free status e.g. Australia, USA and Japan and they could therefore impose non-tariff trade bans on New Zealand's agricultural exports until we achieve this status. At the present time New Zealand is divided into Tb vector risk areas [VRA] and Tb vector free areas [VFA]; the great majority of cattle and deer found to be infected with Tb come from VRAs which cover about 24% of the country. Elimination of Tb from these areas, once thought to be a simple matter of testing and time, is extremely difficult because of the wildlife reservoirs and vectors and despite control efforts it has not yet been possible to wipe out Tb infected possum populations or impose a cordon-sanitaire around these herds [Coleman and Livingstone, 2000].

1.3] Possum Control

Ever since their introduction to New Zealand possums have been caught and killed, initially for their fur, then in response to their depredations on horticulture and the natural environment and more intensely and urgently as they have become a threat to the agriculture and economy of the country. In 1992 the New Zealand government set up a National Science Strategy Committee [NSSC] for the Control of Possums and Bovine Tb as a ministerial committee to "manage and support research to attain the goals of developing control methods to eradicate Tb as an animal disease problem and to protect conservation values from possum damage" [Possum and Bovine Tb NSSC Report, 1992, 93, 94]. This research has encompassed a number of broad areas but in particular,

biological control. This was seen to be long term and conventional methods remain the basis of control at present.

1.3.1 Conventional control

Control by trapping and poisoning has taken place formally under the auspices of, first the Department of Internal Affairs, then the Forest Service and Animal Pest Destruction Boards and, since 1987, the Department of Conservation. The programme has been taking place since 1947 and despite evidence of high numbers of animals being culled [there were between 1 to 3 million skins exported annually during the 70s and 80s], the population has continued to increase in number and continues to disperse into new areas [Cowan, 1990]. "It appears that harvesting has maintained the population in the proliferative phase" [Kerle, 1984].

Conventional control has used traps and cyanide bait, and since 1954 the poison 1080, sodium monofluoroacetate [originally derived from the eucalypt species *Gastrolobium* and *Oxylobium*] has been extensively used on the ground, in bait stations and, most importantly, in aerial applications in carrot or pellet bait. It has been shown that 95% of possums encountering a correctly prepared 1080 bait should eat it and die [Morgan, 1990], however, results are usually not of this order due to factors such as learned and non learned aversion, incorrect poison dose in bait, failure to encounter bait [Kelton, 1994]. Alternative poisons have been and are continuing to be developed and/or assessed in possums and at present there are six compounds registered for possum control: cyanide, the anticoagulants pindone and brodifacoum, a bait containing cholecalciferol, based on the possums' susceptibility to changes in calcium level and phosphorus [Eason *et al.*, 1993]. At present aerial 1080 campaigns are the most successful way of sharply decreasing the numbers of possums in high-density areas, the necessary initial knockdown phase of population control, although public acceptance of such programmes is decreasing [Morgan and Hickling, 2000].

Both eradicating the remaining possums from an area after the knockdown phase and maintaining the population at a sufficiently low level to attain the desired goals for that area, are problematic. Follow-up trapping and maintenance trapping programmes are

labour intensive and therefore expensive. Problems exist also in the low numbers of skilled trappers available and the difference in attitude required for conservation and trapping for skins or bounty [Marshall, 1993]. Of interest here are the results of trials with an infrared camera and DPSG [Differential Global Positioning System]. In an eradication programme on Rangitoto Island the conventional system of aerial 1080 poisoning was used for knockdown and an estimated 90% of possums were killed. Follow up ground hunters believed they had eradicated all possums but the IR camera identified at least 50 possums remaining in three areas [Livingstone, 1994]. Possums also re-colonize areas by both breeding and dispersal where they have been either eradicated or where density has been lowered.

The cost of conventional control is high- it has been estimated that to eradicate possums from areas of greatest conservation value and decrease incidence of Tb to acceptable levels in the worst-affected areas would cost $NZ1 billion [Parliamentary Commissioner for the Environment, 1994]. This is well above what the government, farmers and land care agencies spend at present on possum control.

1.3.2 Biological control.

Because of the problem of continuing and escalating cost and the decreasing public acceptance of large scale poisoning operations [Morgan, 2000] some form of biological control is seen as the only long-term solution to the problem of possum numbers. Biological control in the classical sense is the control of the numbers of one species by another. The NSSC has interpreted biological control as using biological not chemical or physical means to reduce possum numbers rather than in the classical sense above [Jolly 1993].

Whereas ecological studies had been on-going since the early 70s [Fitzgerald and Gibb, 2001] in the early 90s little was known about the physiology of the possum and other marsupials; there were few or no possum specific reagents with which to investigate reproductive or immunological physiology and marsupial-specific processes had not yet been elucidated [Buddle *et al.*, 1995]. The pathogens and parasites that infected possums in New Zealand were known from a few studies in a few discrete areas only

[Burtton, 1975, Bowie and Bennett, 1983]; viruses were not known at all. Systematic surveys of ecto- and endoparasites of *T. vulpecula* were initiated in New Zealand and a search for a possible control agent in Australia was also undertaken. The results of these surveys will be discussed in Section 4 of this introduction.

Comprehensive reviews of the results of the research programmes proceeding under the auspices of The NSSC have been reported in biennial workshops from 1995-2001. Research has moved from the wide-ranging investigations of possum behaviour, physiology, immunology plus the whole country search for pathogens and parasites in the "discovery" phase of the early 90s to research that is more focussed on control: disruption of reproduction by immunological and non-immunological methods, immunological barriers to the delivery of vaccines or biocontrol agents, self-disseminating systems for a biocontrol agent. Details of current research into control strategies are reviewed in Biological Management of Possums [2001].

Two pieces of research, which may have future associations with this project, indicate the nature of current work. In immunologically based fertility control studies, zona pellucida proteins, [the coating surrounding the egg in mammals] ZP2 and ZP3, have been used to immunize possums and the resulting antibodies have reduced fertility in female possums [Duckworth, 1999]. Possum-specific regions of these proteins that correlate with infertility are being used in fertility trials to assess their species specificity and effect on fertility [Harris *et al.*, 2001]. There is work underway to express these ZP antigens, alone or as dual constructs with sperm antigens, in transgenic plants which can then be used as bait [Cowan, 1999, Graham-Rowe, 2000]
Research on reduction in fertility, which is independent of an immunological response, uses gonadotrophin-releasing hormone, conjugated to a toxin to target and destroy gonadotrophin secreting cells in the possum pituitary. *In vitro* and some *in vivo* studies have been carried out [Eckery *et al.*, 2001].

1.3.3 Pathogens of Possums

The internal helminth parasites of possums are considered in Section 4. The most important bacterial pathogen of possums is *M. bovis*, as discussed in Section 2.4.

Reasonably large numbers of possums are also infected with *Leptospirosis interrogans balcanica* in the North but not the South Island; the effect on the health of these possums is slight. Other bacterial and fungal infections have been reported but the incidence is low and the population effects negligible [Cowan *et al.*, 2000].

The search for a disseminating vector system for biocontrol agents has led to an active search for possum specific viruses that could act in this role or cause disease themselves. In 1995 a new disease of possums, "wobbly possum syndrome" was discovered [Mackintosh *et al.*, 1995]; it was transmissible between possums and caused a high rate of mortality, at least among captive possums. This virus has been characterized as a Borna-type virus [O'Keefe *et al.*, 1997]. Although some of the neurological symptoms are similar to a series of cases in the Sydney Zoo Registry causing high mortality they are not now considered the same disease [Obendorf *et al.*, 1997]. A transmissible acute enteritis found in a captive possum colony was also thought to be viral in origin; however, this virus has not been isolated [Zheng *et al.*, 1999]. Adenoviruses, corona virus particles and herpes viruses were identified in intestinal contents [Rice and Wilks 1995] and an adenovirus [Possum adenovirus type1] has been isolated and characterized [Thomson and Meers, 2001]. Herpes virus antibodies have been found but as yet a possum-specific virus particle itself has not been isolated [Zheng, 2001]. A list of potential viral and bacterial pathogens of brushtail possums from a recent survey of Australian possums is appended, see Appendix 1.

Although some are pathogenic it seems unlikely that any of these viruses could act as an efficacious biocontrol agent similar to the rabbit haemorrhagic disease [RHD] virus of rabbits. Certainly some viruses are still being investigated as possible vectors for a biocontrol agent [Thomson and Meers, 2001, Zheng *et al.*, 2001].

1.4] Possum Parasites

From the beginning of the NSSC's work on biological control of possums the search for pathogens or parasites has been a high priority area [Atkinson and Wright, 1993]. A few studies had been done on the micro fauna of New Zealand's own possums but only

very local knowledge was available. The parasite fauna of Australian possums had been fairly extensively documented [Presidente, 1984] but it had not been investigated with the aim of finding a possible pathogenic control organism. It was also intended to widen the search to the phalangerid cousins of the possum, e.g. the cuscuses and *Wyulda* in Papua New Guinea and possibly even Indonesia [Atkinson and Wright, 1993]. The search for a new pathogen further afield than Australia has now been terminated because of the perceived danger of finding an organism that might be harmful to Australian native marsupials and possibly to indigenous fauna in New Zealand also.

It was envisaged that such an organism could be of use in one of two ways: 1) as a biocontrol agent in the classical sense causing morbidity or mortality in a naïve population with low resistance or 2) as a vector for the introduction and expression of foreign DNA which would decrease the fitness or fecundity of the invaders.

1.4.1 Parasites of possums in Australia.

In Australia the parasites and diseases of the *Trichosurus* species were reviewed and catalogued by Presidente in Possums and Gliders [1984]. Most parasites were reported as incidental findings of disease in captive animals or as descriptions of new species, but their distribution, prevalence and significance for the health of their hosts was also recorded where possible. It was noted that the number of parasites species normally found in or on non-captive possums was small. The occurrence in both Australia and New Zealand was noted and it was seen at this stage that New Zealand possums were infected with a smaller range of species, of both ecto- and endoparasites.

A detailed compilation of reported ecto- and endoparasites of both *T. vulpecula* and *T. caninus* in Australia and New Zealand was undertaken in the early 90s [Viggers and Spratt, 1995]. This was to provide a basis from which investigation of biocontrol agents could proceed and indicate organisms that might be of interest for importation into New Zealand. A full list of endoparasites is given in Appendix 1. As well as this review a new search was undertaken; a wide ranging study from autopsied animals was carried out at a number of different locations in Australia obtaining definitive information on parasites from a range of habitats across the entire geographic range of *T. vulpecula*'s

country of origin. Also a small sample of the congeneric species, *T. caninus* [106] and *T. arnhemensis* [10] were sampled in the locations in which they occur and where possible [these animals are not as numerous or wide spread as *T. vulpecula*]. Possums in a monitored population who died of natural causes were investigated to try and determine the causes of their disease [Obendorf *et al.*, 1997].

This survey found no new species in *T. vulpecula*, 12 of the 29 described helminths species and 22 of the 37 known ectoparasites were seen. Two new nematode species were, however, found in *T. caninus*; these are the Metastrongyloidea *Marsupostrongylus minesi* and Spiruroidea *Gongylonema* spp. Of these, the lungworm *Marsupostrongylus minesi* is highly prevalent in some locations and causes pulmonary consolidation and granuloma formation; this has been a candidate for consideration as a disease causing organism in *T. vulpecula*. A list of pathogens causing morbidity or mortality in free-range possums is shown in Appendix 1.

1.4.2 Parasites of possums in New Zealand

The NZ possum population derives from a small founder population of 200-300 possums and the entire range of parasites found in the Australian population is not represented in New Zealand. Until recently the parasite fauna was not well known and had not been systematically surveyed. Prior to 1975 two species of ectoparasitic mites, *Trichosurolaelaps crassipes* and *Atellana papilio* had been found on possums [Pracy and Kean, 1968] who had also found tapeworms in the possum gut, classified as *Bertiella trichosuri* [Khalil, 1970]. *Trichostrongylus colubriformis*, the sheep gut nematode, had also been reported [Sweatman cited in Cowan *et al.*,2000 p82].

An early investigation of intestinal helminths of a small sample of possums from a pine forest near Taupo, New Zealand found *Parastrongyloides trichosuri* for the first time in New Zealand at a prevalence of 100%, along with *Bertiella* and *Trichostrongylus retortaeformis*, from rabbits [Burtton, 1975]. A later study of a larger sample of possums the Waitahuna forest, Central Otago, New Zealand, found that 35% of possums were infected with *T. colubriformis*, no other nematodes of possums were found. Three

species of Acarine mite were found to infest nearly all [98%] of possums [Bowie and Bennett, 1983].

A large-scale survey of the parasites present in the NZ population was undertaken in response to the recommendations of the NSSC for research on biological control. The 16 original sites where possums were released were chosen in order to increase the chance of sampling the widest possible parasite range [Heath *et al.*, 1997]. The survey methodology and parasitological methods are covered in Heath *et al.*, [1997] and Stankiewicz *et al.*, [1997]. Full results of the survey are available on a possum database [Possum Access Database, AgResearch, 1996] and in Stankiewicz *et al.*, [1997, a, and b]; they are summarized in Heath *et al.*, [1997] and in Table 1.1 and Fig 1.4.

It is now certain that New Zealand possums lack many of the parasites present in the Australian population of origin and in many areas have not been parasitized by the full range of species present in New Zealand, for instance, neither of the nematodes *Parastrongyloides trichosuri* or *Paraustrongylus trichosuri* are found anywhere in the South Island except in Riverton, one small area in the far south.

1.4.3 Parasites as biological control agents.

With New Zealand's limited parasite and pathogen fauna it would appear that there is ample scope for introducing one or more parasites from Australia. The suggested species have been *Adelonema trichosuri*, a nematode parasite with a high prevalence in parts of Australia, and *Marsupostrongylus minesi* found in *T. caninus* where it causes inflammation of the lung and is more pathogenic than other parasites. This latter was not found in *T. vulpecula* in the recent survey but is reported to be pathogenic in this species also [Obendorf *et al.*, 1997]. Another suggestion has been the *Chlamydia psittica* organism that causes infertility in koalas and has significantly reduced the size and fecundity of populations in which there is a high incidence. There is no record of this organism infecting possums [*ibid*].

The importation of a new parasite or pathogen is fraught with ecological and regulatory difficulties, as witnessed by the recent Rabbit Haemorrhagic Disease [RHD] importation

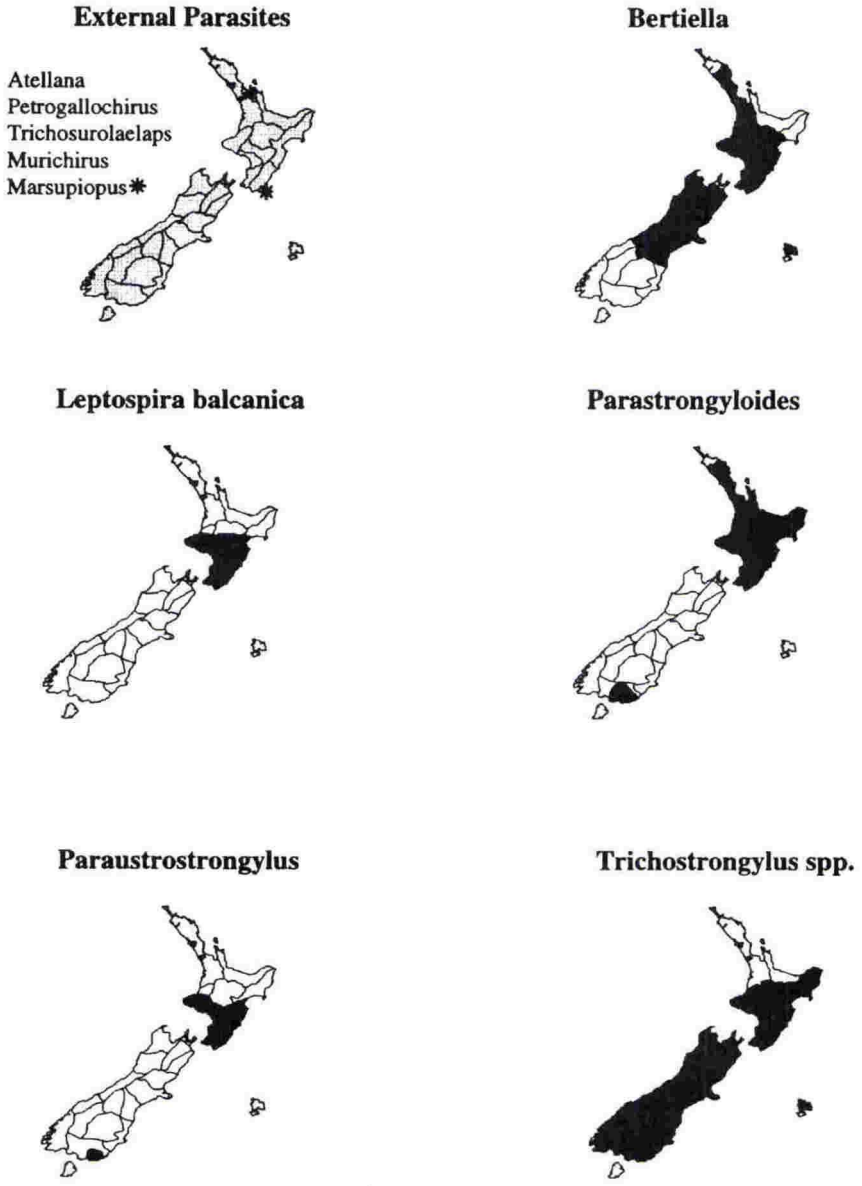


Fig 1.4 The distribution of possum parasites in New Zealand from the large possum survey: data obtained by sampling approximately 200 possums from each of 16 regions where possums had been originally released. (reproduced from Heath et al 1997 – with permission)

Table 1.1 Prevalence (%) of Endoparasites of possums from the sampling sites from the large possum survey.

Sites	*Eimera Spp.* (Protozoa)	*Bertiella trichosuri* (Cestode)	*Parastrongyloides*	*Paraustrostrongylus* (Nematode)	*Trichostrongylus* sp.
North Island*					
Hawkes Bay	10.1	2.9	15.8	12.8	32.4
Wanganui	23.0	0.5	55	2.4	58.9
Castlepoint	21.4	5.7	63.2	21.2	33
Northland	21.8	4.4	60.2	0	30
Orongorongo	5.2	3.0	60.4	51.1	37.9
Palmerston North	4.0	2.4	54.9	70.3	24
Paraparaumu	20.0	0	51.9	53.8	23.1
Mean - Northland	**15**	**2.7**	**51.6**	**30.2**	**34.2**
South Island+					
Banks Peninsula	6.1	27.5	0	0	55.3
Hokitika	6.3	0.6	0	0	26.6
Nelson	9.5	24.3	0	0	19.4
Dunedin	10.3	0	0	0	35.7
Invercargill	3.8	0.0	14.0	1.4	34.3
Mean - Southland	**7.2**	**10.6**	**-**	**-**	**34.3**
Offshore Islands§					
Kawau	16.7	5.2	15.5	0	0
Chatham	10.9	3.6	0	0	8.1
Stewart	4.6	0	0	0	0
Overall Mean for infected areas	**11.6**	**5.8**	**43.3**	**26.6**	**32.2**

* Data from Stankiewicz *et al.* 1998
\+ Data from Stankiewicz *et al.* 1997
§ Data from Stankiewicz *et al.* 1997b.

into New Zealand [Thomson and Clark, 1997], and some prior evidence of efficacy would be needed. Although biocontrol of invertebrate pests, particularly insects, by the introduction of parasites has had a number of important successes [Cameron *et al.*, 1989] biocontrol of vertebrate populations has not been so successful, with the notable exception of rabbit control by myxomatosis and latterly RHD [Fenner and Fantini, 1999].

It is generally assumed that parasitism per se is deleterious for a population as a whole [Anderson, 1978]. Parasites undoubtedly have a detrimental effect on some individuals in a population; in all possums surveys listed the overdispersed distribution of parasites has been seen, with one or two emaciated or dead animals found with very heavy parasite loads [Heath *et al.*, 1997, Viggers and Spratt, 1995, Obendorf *et al.*, 1997]. There is always the caveat: have the parasites caused the morbidity or poor condition permitted higher parasite loads? Whether or not parasites have an effect on the entire population that is large enough to affect density substantially is not entirely resolved. This effect, of parasitism reducing population density, has been modelled fairly extensively [Anderson and May, 1978, May and Anderson, 1978] and data from experimental infections of rodents under laboratory conditions has supported the models [Scott, 1987, 1990]. The evidence from studies of populations under natural conditions is less clear cut [Spratt, 1990, Singleton and Chambers, 1996, Grenfell and Gulland, 1995].

Evidence from the long running and extensive study on the red grouse [*Lagopus lagopus scoticus*] and its parasite the caecal threadworm [*Trichostrongylus tenuis*] has shown that parasitism is a sufficient cause of cyclic population fluctuations [Hudson *et al.*, 1998]. And study of the invasion of the house finch [*Carpodacus mexicanus*] by the bacterial parasite *Mycoplasma gallisepictum* has given the first clear demonstration of density dependent regulation of a host population, albeit by a microparasite [Hochachka and Dhondt, 2000].

There are some preliminary models of the effect of parasites on possums [Roberts, 2002] and two studies have been done recently in which the parasite burdens of natural free-ranging populations have been manipulated with the aim of providing data and

possibly a direct answer to the question. In one study a small group [n=8] of adult female *T. caninus* were given anthelmintics to remove intestinal parasites, and their reproductive success compared to a control group. All animals in the treatment group became re-infected between parasite removals and there was no significant difference in the numbers who bred successfully compared with untreated animals [Viggers *et al.*, 1998].

A larger study of *T. vulpecula* was carried out in the Aorangi State Forest National Park, Wairarapa, an area with the full range of parasites present in the New Zealand population. This aimed at comparing survival and fecundity of parasitized possums with a group from which parasites had been removed, again with an anthelmintic drench. It proved impossible to prevent re-infection of the treated possums between treatments at two-monthly intervals although the treated and control populations were separated geographically and sites were located to avoid any possibility of overlap in home ranges [Ralston *et al.*, 1998]. This question remains at present unanswered.

Amongst the organisms recorded from possums so far there does not appear to be a candidate that might produce an epidemic leading to an immediate substantial reduction in numbers but adding one more parasite to a population with a very high density could incrementally increase the parasite load decreasing fitness and thus reduce population numbers towards a more "Australian" level. Modelling has shown that in a natural population, if there is a density dependent relation between host and parasite, these can remain the key regulator of host growth despite only a small number of host deaths being directly attributable to the infection and morbidity due to parasites appearing to be rare [Anderson, 1995].

The second use for a parasite, namely as a vector of some foreign genetic material, still appears a rational area of study with the nematode parasites seeming to warrant further investigation.

1.4.4 Possum Specific Nematodes

Two nematodes present in the New Zealand population have been identified as possible potential vectors for a biological control agent; these are *Paraustrongylus trichosuri* and *Parastrongyloides trichosuri*, both appear to be specific for *T. vulpecula* in New Zealand and trichosurus species only in marsupials in Australia

Paraustrostrongylus trichosuri

Phylum: Nematoda

Order: Trichostrongyloidea

Family: Herpetostronglidae

Genus: *Paraustrostrongylus*

species: *trichosuri*

This was first described as a new genus and species by Mawson in 1973. Species of the nematode family Herpetostronglidae occur in the small intestine of Australian marsupials; the family shows links with South American fauna and is possibly a parasite of the original Australian immigrants [Beveridge and Spratt, 1996]. The genus *Paraustrostrongylus* occurs in diprotodont marsupials with a close evolutionary parallel between hosts and parasites. Congeneric species have been described from potoroo, the bettong *Bettongia gaimardi* and one species is found in a eutherian mammal *Rattus fuscipes*.

Adult *Paraustrongylus trichosuri* are red sinistrally coiled worms, males are approximately 5mm in length, females are 6mm. The life cycle of the species follows a similar pattern to other Trichostrongyloid species with a faecal/oral infection route. Adult worms surgically implanted into a parasite free possum started to produce eggs from 14-21 days after implantation. Eggs are from 40-75 µm; when passed in the faeces they develop into free-living larvae that grow and undergo two moults to become infective third stage larvae which show a greater degree of tissue differentiation and are ensheathed. These larvae are negatively geotropic and thigmotropic and stand up on the ends of leaves and grass aiding ingestion by the browsing host. The third stage larva is parasitic, it exsheaths in the gastro-intestinal tract of the possum to become the fourth stage larvae, it then develops into the adult. Adult worms, shown in Fig 1.5, are found coiled around villi of the duodenum, jejunum and sometimes, proximal ileum; they are

1mm

Fig 1.5 *Paraustrostrongylus trichosuri*, adult parasitic worms from the small intestine of a possum

also found unattached in the mucus and intestinal lumen, coiled and uncoiled and moving through the contents [Heath *et al.*, 1997, Tempero *et al.*, 1997].

Paraustrongylus trichosuri is found in the lower half of the North Island, and in a small area in Southland [Fig 1.4]. Prevalence figures have varied in the different areas studied: in the Wanganui population it was 2.4% [Stankiewicz *et al.*, 1997a], 82% was recorded in the Orongorongos [Charleston and Cowan, unpublished]; in one population [Palmerston North] studied for seasonal and spatial effects prevalence averaged around 70% for animals over 1 year of age in all seasons. The overall prevalence in infected areas was 27%, see Table 1.1. A few individuals have a very high number of worms but except for these the intensity of infection is generally low [Stankiewicz *et al.*, 1998]. There is no known pathological effect of infection with *Paraustrongylus trichosuri*.

Parastrongyloides trichosuri
Phylum: Nematoda
Order: Rhabditoidea
Family: Strongyloididae
Genus: *Parastrongyloides*
species: *trichosuri*

Parastrongyloides was first distinguished from strongyloids by Morgan in 1928, the presence of males in the parasitic generation being the distinguishing feature. *P. trichosuri* was first described by Mackerras in 1959 who described the morphology and life cycle of the species.

The life cycle of *P. trichosuri,* shown in Fig 1.6, consists of a parasitic and a free-living generation. The parasitic adults, fine whitish worms, live in the intestine of the possum and are thought to inhabit the crypts at the base of the villi in the duodenum, jejunum and proximal ileum. Parasitic males are from 3-4mm long with two small round coils at the posterior end obscuring the spicules and gubernaculum; the parasitic female is slightly larger, 4.2-5.2 mm with a straight, somewhat pointed tail; eggs can usually be seen in the uteri. Adults are shown in Fig 1.7. Parasitic females lay oval clear-shelled eggs into the host intestine that start developing and are passed out with the faeces at various stages of development up to and including newly hatched larvae. Larvae

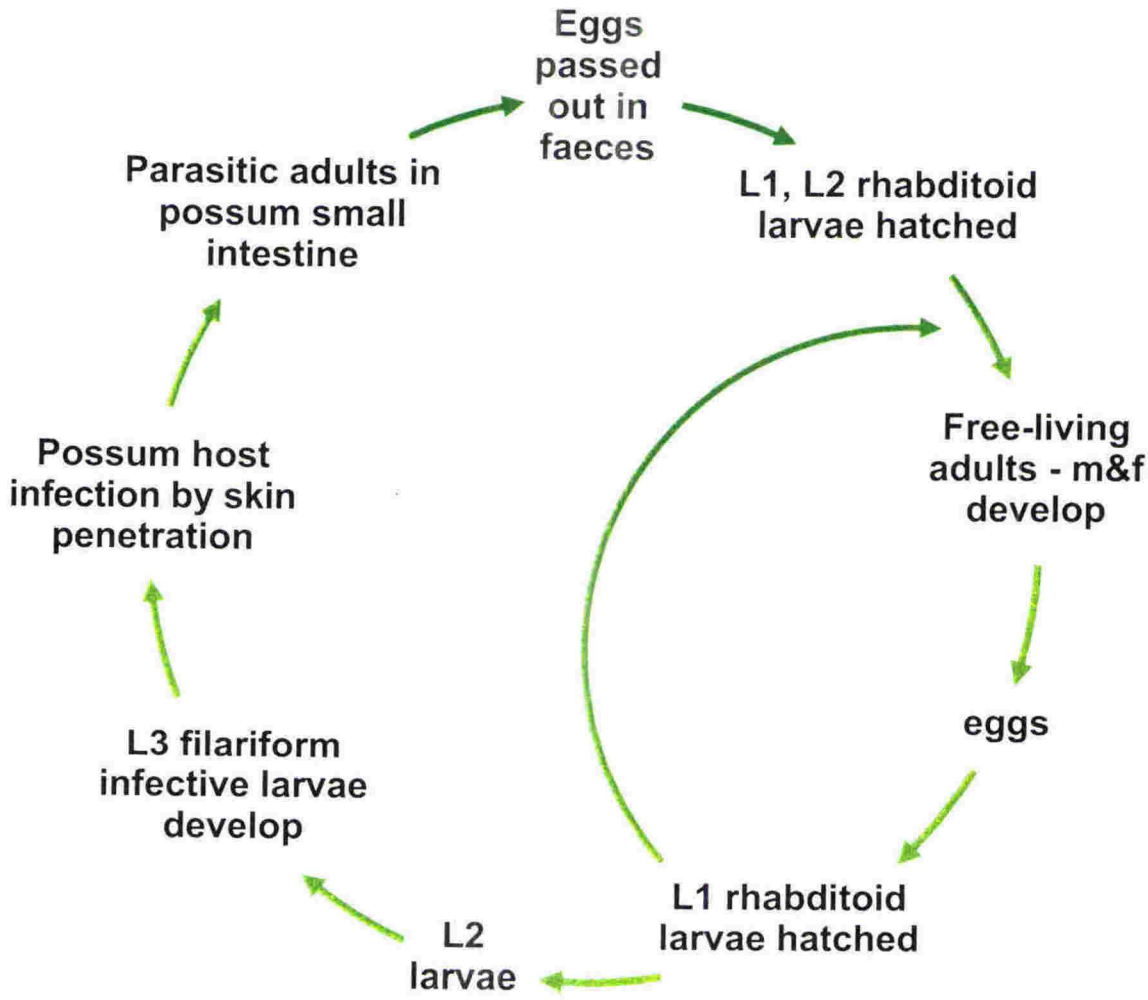
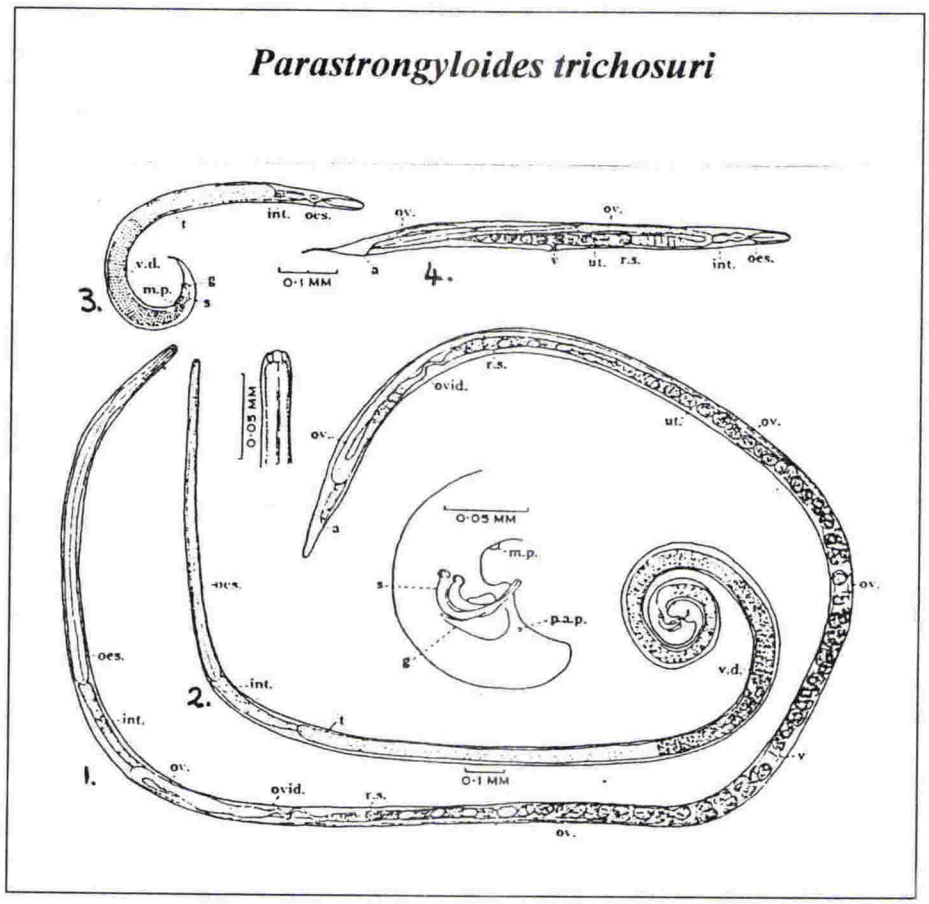
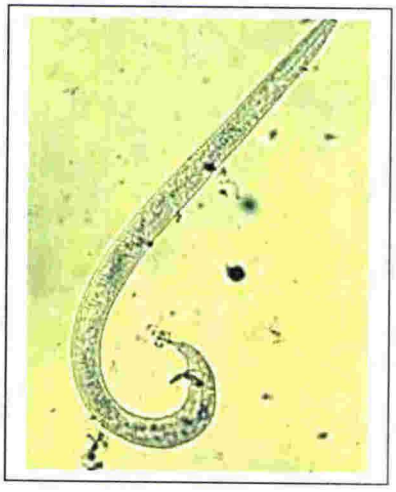


Fig 1.6 Lifecycle of *Parastrongyloides trichosuri*

A

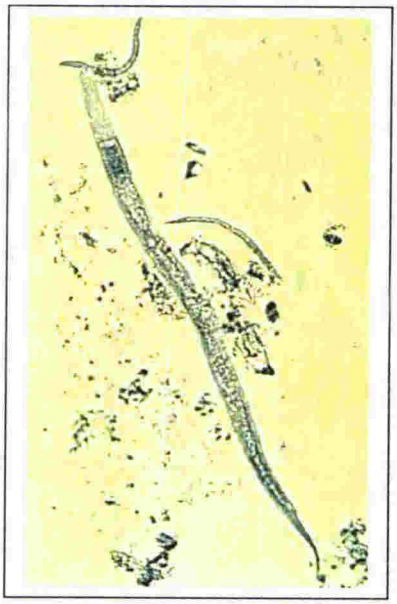


B



0.1mm

C



0.1mm

Fig 1.7 *Parastrongyloides trichosuri.* A] Drawing from the original description of the parasite, Mackerras 1959. 1) parasitic female, 2) parasitic male, 3) free-living male, 4) free-living female.

B] Adult free-living male. C] Adult free-living female

undergo three moults until adult characteristics can be seen in the L4 larvae, at the fourth moult small plump worms with long pointed tails about 0.7 mm long result; these are the free living adults.

Free-living females lay eggs that are slightly smaller than their parasitic counterparts. Some eggs develop into a second generation of free living adults following a similar pattern as described for the eggs from the parasitic females; some, however, follow a different developmental pathway and after a second moult becomes an infective third stage larvae. The long highly motile infective [filariform] larva leave the faecal mass in which they develop and migrate upwards; they also move towards objects with the body temperature of a warm-blooded animal [Stankiewicz, 1996]. They complete the life cycle by penetrating the skin of the host animal and migrating via the lungs to the gut where they develop into new parasitic adults. It is thought that the stimulus to enter the infective developmental pathway is adverse environmental conditions [Heath and Sharma, unpublished]. The continual cycling of the free-living forms allows amplification of the numbers of the infective form.

P. trichosuri is a common parasite found in *T. vulpecula* and *T. caninus* in Australia [Viggers and Spratt, 1995] but not in other marsupials [Spratt *et al.*, 1991]. In New Zealand it is distributed throughout the North Island, except for a small area at the top of Northland, and is present in southeast Southland [Fig 1.4]. Prevalence was 55% in the Wanganui population and the intensity of infection increased with age [Stankiewicz *et al.*, 1997a], 97% from the Orongorongo population [Charleston and Cowan, unpublished]. Prevalence data from the large possum survey are shown in Table 1.1; the overall prevalence in infected areas was about 43%. Seasonal and spatial effects studied in the Palmerston North possums showed prevalence averaged around 70% for animals over 1 year of age in all seasons. *Parastrongyloides* is thought to infect all young possums during their first year of life [Heath *et al.*, 1997].

1.5] Rationale and overview of this study.

1.5.1 Advantages of *Parastrongyloides trichosuri*.

The concept of a nematode parasite that could be genetically modified and would act as a self-disseminating vector to spread a gene or gene product throughout the possum population resulting in a decrease in fecundity or survival is innovative. So far, to our knowledge, there has been no other proposition to harness the new technology of genetic modification to create a transgenic organism that would attack its host in a novel way by using a molecule from a completely different species as a weapon. This proposal was included in the Biological Control of Possums programme in 1994.

Of the organisms available, *P. trichosuri* has some unique attributes that give it considerable potential to act as such a vector. These advantages are:

1) The parasite is already present in New Zealand possums; this obviates the difficulties of importing a new organism. The areas where parasites are found at present are shown in Fig 1.4 but there are also areas where the parasite is not present and these can, and indeed are being used to investigate the dynamics of the parasite's spread out from an existing infected area [Ralston *et al.,* 1999] and throughout a naïve population [Ralston *et al.,* 2001].

2) *P. trichosuri* is species specific. Specificity is the *sine qua non* of such a biocontrol programme. Domestic animals are routinely searched for parasites e.g. in abattoirs and for public health reasons, e.g. during the eradication of the hydatid tapeworm in dogs. Dead wildlife are scrutinized occasionally and their parasites recorded. At no time have either possum specific parasite been reported from any animal other than possums in the hundred and fifty years since their introduction; both nematodes have a very characteristic appearance and it would be difficult to have missed the bright red *Paraustrostrongylus trichosuri* [Fig 1.5]. Some species specificity studies have been done at Wallaceville. New Zealand white rabbits developed antibodies to the parasites after sub-cutaneous infection but no worms were recovered from their small intestines [Heath *et al.,* 1997]. Rats, mice

chickens, guinea pigs and ferrets have been infected by sub-cutaneous injection of infective larvae but no worms were recovered or characteristic antibodies found in the serum of any other species [Ralston, personal communication]. There is considerable confidence in the host specificity of this parasite.

3) The parasite has an unusual life cycle with a free-living life stage; this has allowed it to be established and maintained in laboratory culture [Gruenberg and Bisset, 1997]. *P. trichosuri* eggs from a parasitic female in the gut of a monospecifically infected possum are passed in the faeces and recovered by salt flotation. They are cultured on agar plates to which a few drops of possum faecal filtrate have been added; faeces from a non-infected possum appear to contain bacteria specific to the possum gut or some other unidentified substance necessary for the survival of *P. trichosuri*. Plates are kept at room temperature, 15°-25°C, but eggs can hatch and go through at least one free-living cycle at 10°C, development appears to be arrested at 8°C [Campbell, 2001]. Young free-living adults are transferred to new plates with fresh faecal filtrate; if not transferred the offspring become irrevocably committed to the parasitic pathway and develop into infective filariform larvae that can be used to infect possums. New cultures from different populations are readily set up and old ones have been maintained without loss for seven years.

4) The free-living life cycle stage has the potential for genetic modification. The free-living adults of *P. trichosuri* show morphological similarity to the free-living soil nematode *Caenorhabditis elegans*. This organism has become a model for the study of development and gene function and regulation [Brenner, 1988]. Microinjection of foreign DNA which is expressed in future generations of the progeny is carried out almost routinely in *C. elegans* [Fire, 1986, Stinchcomb, 1985 Mello *et al.*, 1991] and the knowledge and expertise derived from this work can be transferred to similar organisms. Initial experiments with *C. elegans* have established the techniques of microinjection at Wallaceville [Gruenberg and Bisset, 1997].

5) The parasitic phase of the life cycle can spread throughout the entire possum population, it requires no other intermediate host and infective filariform larvae

readily infect possums they come in contact with. They migrate out of the faecal mass and move towards increased temperature, though not towards light [Stankiewicz, 1996] and infect by penetrating the skin although it is also possible to infect possums orally. Parasites inhabiting faeces kept under laboratory or natural conditions in the bush [protected from further contamination] developed infective larvae that were still capable of infecting possums for five to six weeks [Ralston, personal communication]. As stated in Section I.4 these parasites are highly prevalent in possums in infected areas and it is postulated that all possums come in contact with the parasite at least once a year [Heath *et al.*, 1997]. These reasons make *P. trichosuri* a highly favourable candidate for spreading a controlling factor throughout the possum population.

1.5.2 *C. elegans* as a technology development model for *P. trichosuri*

Since the pioneering work of Sydney Brenner in the nineteen sixties, *C. elegans* has been developed as a model organism for studying the biology, cell biology, development and genetics of multicellular organisms. A complete cell lineage diagram has been compiled a neuronal wiring diagram and a physical genetic map are available. Features such as its short life cycle, mode of reproduction, i.e. as self-fertilizing hermaphrodites or out-crossing hermaphrodites with males, its small genome have all contributed to its usefulness in the field of genetics [Riddle *et al.*, 1997]. The sequence of the entire genome is now known [The *C. elegans* Sequencing Consortium, 1998] and there is an extensive catalogue of ESTs available; all this information is readily accessible through *C. elegans* databases [ACeDB at http://elegans.swmed.edu].

The techniques of creation of mutants by transposon insertion or chemical mutagenesis followed by rescue by complementation and more recently by RNAi, interference of gene function by introducing double-stranded RNA corresponding to a specific gene has allowed function to be attributed to a gene. Transgenic animal technology has allowed gene expression patterns to be studied and can be used for gain-of function analysis of gene function. The use of *C. elegans* as a heterologous system for studying the regulation and expression of genes from parasitic nematodes has been advocated as

there is no parasitic system in which this can be done at present [Grant, 1992, Hashmi *et al.*, 2001].

The technique of transformation of the model nematode *C. elegans* started around 1982 with the microinjection of suppressor tRNA into the gonad of an amber mutant with consequent rescue [Kimble, 1982]. Successive improvements in techniques by Stinchcomb *et al.*, 1985, Fire, 1986 and Mello *et al.*, 1991 have led to the routine microinjection of exogenous DNA into the gonad of many strains of the worm in order to characterize the function of cloned genes *in vivo*. Plasmids containing the gene or regulatory sequences of interest and usually some selectable marker are injected via a very fine needle into the central cytoplasmic region of the syncytial gonad of *C. elegans*. The transfected DNA is highly reactive when first injected with the plasmids undergoing homologous recombination to form large extra-chromosomal arrays; it is believed that the plasmids form only one or a few such arrays per cell. The arrays are estimated to be concatamers of from 100 to 300 plasmids with the larger arrays being more stable and more easily inherited than the smaller ones; once established, these structures are then thought to undergo little further change [Mello *et al.*, 1991].

Integration of transfected DNA into the *C. elegans* chromosome can also occur by direct injection into the nuclei of developing oocytes at the position in the gonad where DNA is being packaged and incorporated into ova, around the turn in the gonad [Fire, 1986]. It is possible for whole functional genes to be integrated as up to 7kb of plasmid DNA may be incorporated in this way and stably transfected lines can be obtained. The frequency of integration in this way is very low.

Heritability of extra-chromosomal structures is more frequent but less stable than DNA that is integrated into the chromosome and the arrays are inherited in a non-Mendelian manner. In a number of cases reasonably stable, "semi-stable" transformant lines have been established from transgenic F1 offspring in which a particular percentage of the progeny always receive the transgene; the percentage depends on the nature of the construct being used and varies from 5-95% [ibid]. Expression of the foreign DNA has been shown to be comparable between the integrated and the extra-chromosomal transfections.

In order to facilitate transgenic research with this organism a number of *lacZ* fusion vectors have been developed whereby the 5' region of the gene to be studied is inserted upstream of the reporter *lacZ* sequence or *lacZ* and GFP [Green Fluorescent Protein] [Fire *et al.*, 1990, Chalfie *et al.*, 1994], these are widely used and have been the basis of the plasmids used in our laboratory for microinjection work with *C. elegans* and *P. trichosuri*. It is likely that the transgenic technology developed in *C. elegans* can be applied to P. trichosuri with a reasonable chance of success because of their anatomical similarity and close evolutionary relationship.

1.5.3 Immunological studies of *P. trichosuri*.

The prevalence and occurrence of *P. trichosuri* throughout the sampled possum population has been studied by finding the parasitic adults in the small intestine at necropsy. The monitoring of current infections in captive or trapped animals can be done by finding parasite eggs in faecal samples. Neither method allows the determination of past infection history to distinguish between animals that are immune or those who have never met the parasite. Both of these states are of importance when considering the spread of a modified organism throughout a naïve or previously infected population. Therefore a sero-diagnostic tool was sought.

Immunological studies of *P. trichosuri* were carried out and it was found that partial immunity to the parasite could be created by repeatedly infecting possums with an increasing number of filariform larvae then truncating the infection with anthelmintics [Heath *et al.*, 1999]. This immunity was shown by a reduced response to challenge as measured by worm burden in the small intestine at necropsy and the presence of antibodies in the serum of immunized possums. Immunoblotting larval antigen with possum serum showed characteristic bands in the 65-80 kD region that were seen in seven out of ten immunized possums and two of the ten controls. Further analysis resolved this region into four or five bands with 70% of infected possums showing one or more of these bands. One of these, a band at around 70 kD was the most commonly occurring [Flanagan *et al.* unpublished].

Serum samples from several sites in the large survey were used to examine possums that had been infected with one, two, three or no nematode parasites for the presence of these antibodies. Fig 1.8 shows a set of these results. In areas where *P. trichosuri* is endemic approximately 70% were found to have these antibodies whereas in the South Island where the parasite is not present, none of these antibodies were seen. They appear to be specific for infection with *P. trichosuri* [ibid]. It was considered that the presence of antibodies to this set of proteins detected in the blood of captured possums might be useful as an indication of exposure to the parasite and, in epidemiological studies, for following the spread of a parasite through a population and investigating possible immunity in possums that did not show infections in an endemic area.

These proteins appear to be present only in *P. trichosuri*; possums infected with other nematodes did not show antibodies to proteins of this size. It was thought likely that the presence of systemic antibodies could be correlated with the mode of infection peculiar to this parasite which is by penetrating the skin of the host. In order to do this, the larvae must secrete proteinase enzymes and further enzymes are almost certainly involved in the migration through host's tissues to the gut where they establish. This would expose such proteins to the immune system of the host to a far greater degree than exposure at the immunologically protected mucosal barrier of the gut. Many parasitic helminths have been shown to secrete proteinases in order to invade their hosts, digest host tissue for their nutrition or evade the host immune response. The majority of these proteinases are of the papain super-family of cysteine proteases [Tort *et al.*, 1999]. It is possible and would make sense that the immunogenic proteins of *P. trichosuri* are such secreted proteases.

1.5.4 *P. trichosuri* as a biocontrol vector.

In order to develop this parasitic nematode as a biocontrol vector two things are needed: Firstly, methods of successfully microinjecting the worm and culturing transfected offspring need to be developed; these are discussed in Chapter III. Secondly, a DNA construct is required consisting of a gene that will cause decreased fecundity or morbidity in the possum when expressed by the parasite. Two possible molecules have been described in Section 1.3.2. Such a construct also requires regulatory sequences

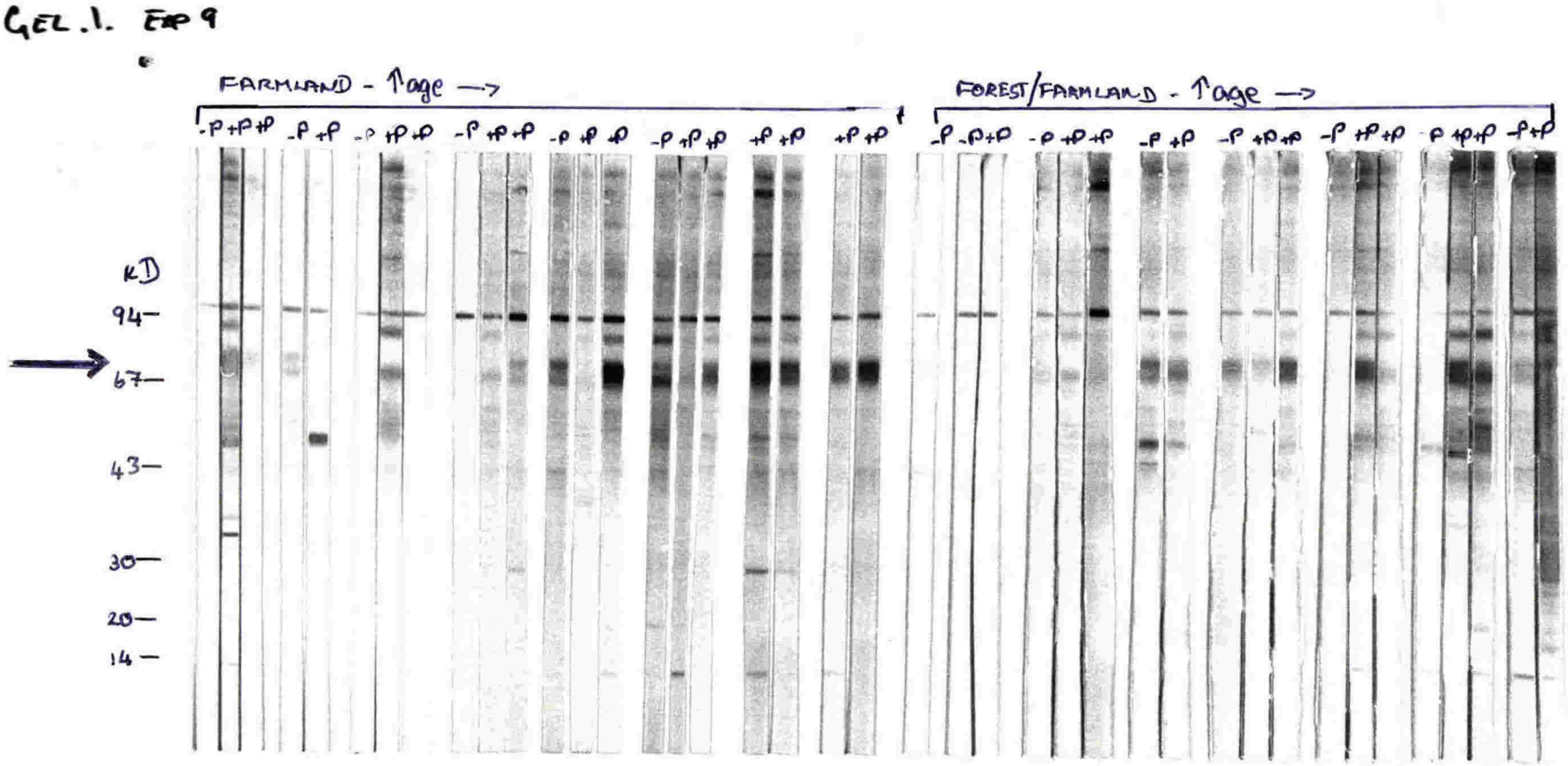


Fig 1.8 Immunoblot of *P. trichosuri* larval antigen blotted with possum serum from possums of different ages and infection status inhabiting forest and farmland in the area of Wanganui, New Zealand. The characteristic antibody band in the 67-80 kD region is indicated → [from Flanagan *et al.*, reproduced with permission]. –P = no current infection with *P. trichosuri* +P = current infection with *P. trichosuri* present. +T = current infection with *Trichostrongylus colubriformis* present

that would control, and, hopefully, maximize gene expression in *P. trichosuri*. The regulatory sequences of a secreted protein that is immunogenic would be a desirable candidate for this part of the construct.

1.5.5 Aims and plan of this project

The aims of this project were to:

1) Identify an immunogenic protein from *P. trichosuri* from among those that had already been indicated as being present, described in Section 1.5.3. It was planned that this would be done by monospecifically infecting possums with *P. trichosuri* and obtaining parasite material from which to isolate this protein.

The individual protein would be detected by its reaction with antibodies in serum from possums infected with *P. trichosuri*. It would be isolated either from excretory/secretory products of cultured parasites or from parasite material itself using protein separation methods such as 1D and 2D electrophoresis. It would then be identified by amino acid sequencing and comparison with known peptides in the databases – **Chapter II**.

2) Isolate the gene for the immunogenic protein by standard molecular biological techniques. The peptide information obtained in 1) would enable an oligonucleotide probe to be constructed, which would then be radioactively labelled and used to screen an existing *P. trichosuri* genomic library. Clones containing all or part of the gene and would be sequenced and this sequence compared with sequences in the databases to attempt to determine the structure and function of the gene product. This is described in **Chapter III**.

3) Prepare a DNA construct for microinjecting into *P. trichosuri*. A DNA region upstream of the start codon for the gene would be amplified by PCR from the library clone; this fragment containing the putative promoter region would be cloned into a vector immediately in front of a reporter gene and the construct microinjected into the gonads of adult *P. trichosuri*. It was hoped that such a vector would succeed in modifying the parasite and give rise to transgenic offspring. This is described in **Chapter IV**.

4) Make a recombinant protein from the gene for use in immuno-diagnosis in ecological studies with *P. trichosuri*. It was planned that this would be done by making cDNA from the parasite, cloning the coding sequence of the gene into an expression vector and expressing the protein in *E. coli*; this is described in **Chapter V**.

The scope of this project, as it relates to the overall biological control of possums, is delineated in Fig 1.9.

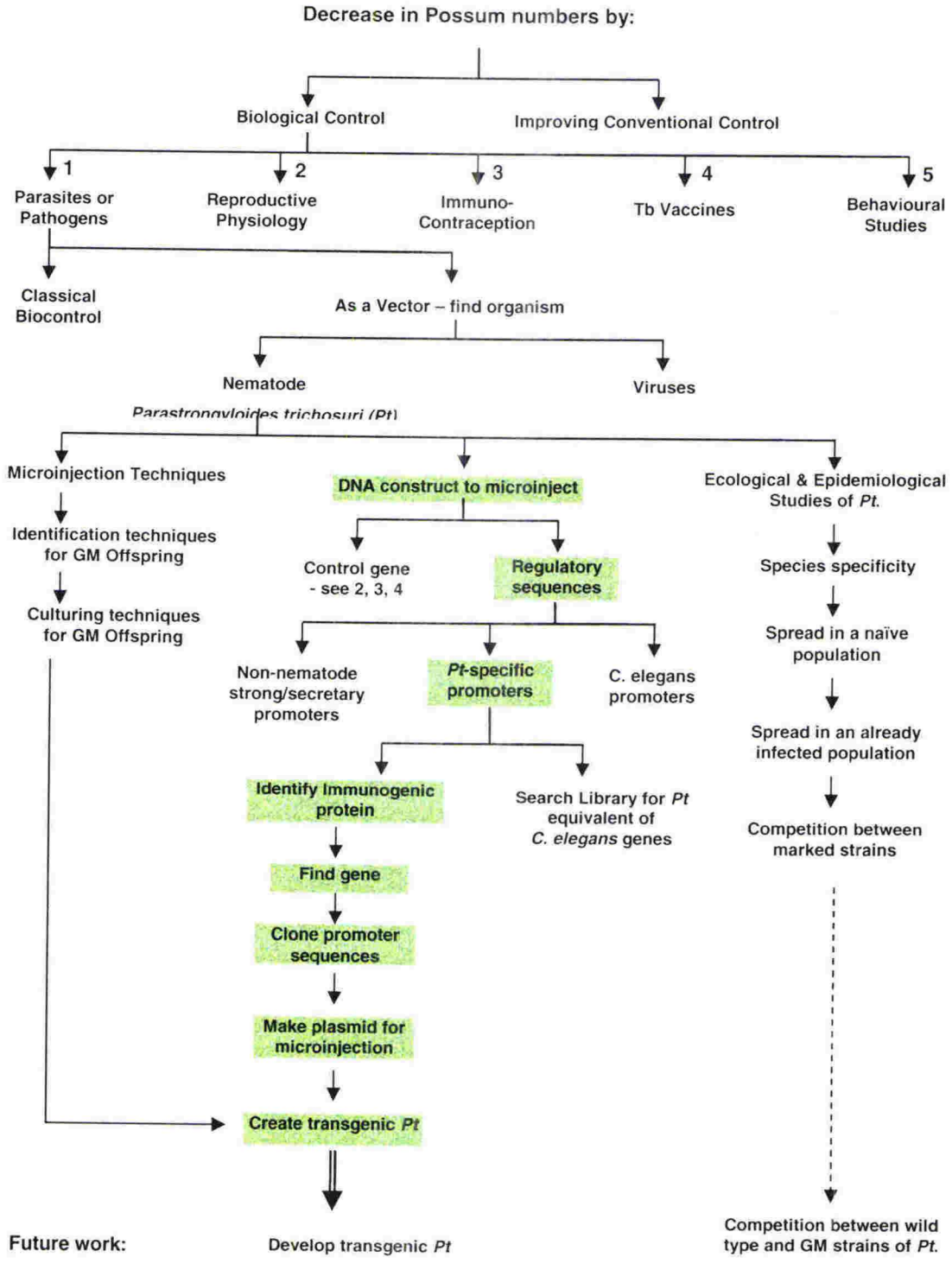


Fig 1.9 The scope of this project and its relationship to the whole Biological Control of Possums project.

Chapter II Identification of the Immunogenic Protein

Introduction:

There are many molecules associated with parasites that elicit an immune response in their hosts and attempts to isolate and characterize them have been carried out since the mid 1980s. This has been driven largely by the need to find candidates for vaccines to protect people and animals from the highly prevalent diseases resulting from helminth parasitism [estimated at around 3.5 billion, WHO report, 1996]. The isolation, molecular characterization and cloning of specific antigens is seen as a necessary prerequisite for accurate diagnosis, vaccine manufacture, both protein and DNA, or therapies aimed at reducing the pathology of the disease [Knox, 2000].

Antigens have been characterized from many of the important helminth parasites of humans and agricultural animals. Two examples from our laboratory have involved identifying the 45W antigen from *Taenia ovis* [Johnson *et al.*, 1989] and the Eg95 antigen from *Echinococcus granulosus* [Heath and Lawrence, 1996]; this has led to the manufacture of vaccines giving protection against these parasites. However, many of the antigens that are identified by antibodies from immune animals in this way in fact play no part in protection of the host against infection from these organisms.

In most cases the antigens are identified by screening Western blots of parasite proteins with serum from infected, and/or, immune animals; the protein is isolated then identified if possible by sequence comparison with known proteins in the databases. The gene can be found by screening cDNA expression libraries with polyvalent antibodies or, cDNA or genomic libraries with oligonucleotide probes from homologous genes. Positive clones are sequenced and the gene expressed as a fusion protein for use in the procedures mentioned above.

It was decided to attempt to isolate and identify an immunogenic protein from *P. trichosuri* using the above approach. The initial hypothesis was that there was a group of antigenic proteins secreted by infective L3 larvae of *P. trichosuri* as they penetrated the skin of the

host; the most commonly occurring of these, an approximately 70 kD protein, became the target for identification. In order to identify the immunogenic protein a "large" supply of parasitic material was required as this protein was one small component of a complex mix. L3 filariform larvae had been used as an antigen in the immunoblots that had first identified these proteins and this life-cycle stage is more easily obtained than others, so large amounts of this material was cultured. The group of 70kD proteins are detected by antibodies in *P. trichosuri* infected possums [described in Chapter I.5.3, Fig 1.8], so methods for detection of the immunogenic protein were re-established. Attempts to demonstrate that the protein was secreted were undertaken; and methods for isolating the protein from larval antigen described. Parasitic adults were also used for some investigations, although early in the project it was considered unlikely that the immunogenic protein would be present in this stage. All parasitic stages require the involvement of the possum host and all procedures using live possums were carried out with approval of the Wallaceville Animal Ethics Committee.

Methods:

2.1] Possums and parasites.

2.1.1 Possums- care and handling procedures

Possums were live trapped from the Upper Hutt or Whiteman's Valley area, New Zealand, or from the Lewis Pass area of the South Island and transferred to Wallaceville Animal Research Centre, Upper Hutt. They were kept either as an initial source of parasites or as hosts for a *P. trichosuri* monospecific infection. Animals were acclimatized for one to two weeks as described in Heath *et al.*, [1999], then kept in individual wire cages 70 x 70 x 80 cm containing wooden nest boxes of 30 x 24 x 24cm. They were fed a selection of fruit and bread daily with water and cereal-based pellets were freely available at all times. They were observed daily by animal care staff for food consumption, signs of diarrhoea and general health.

Possums were anaesthetized for all procedures; an intramuscular injection of 50 mg/kg of Ketamine hydrochloride [Phoenix Pharm, Auckland, NZ] was given for anthelmintic drenching, 25 mg/kg Zoletil 100 [Virbac Laboratories, Auckland, NZ] for bleeding or transdermal infection. Blood samples from some possums were taken from the jugular vein or by cardiac puncture just prior to euthanasia. Euthanasia was carried out after anaesthesia by intra cardiac injection of sodium pentobarbitone. Possums, other than those which were used as an initial source of parasites, had all internal parasites removed with two doses of oral anthelminthics, a mixture of levamisol at 37.5 mg/kg and albendazole at 23.5mg/kg [Arrest® Ancare, Ltd] given one week apart. This dose rate is five times the recommended dose for sheep but previously had been found to be necessary for the removal of all parasites, especially *Paraustrostrongylus trichosuri* [Ralston *et al.*, 2001a]. Drench was administered by syringe with 3cm of soft plastic tubing attached and administered directly into the oesophagus; this effectively reduced the risk of drench inhalation or non-ingestion. Two consecutive faecal egg counts of zero, at least two weeks apart, were taken to indicate that the possum was parasite free.

2.1.2] Faecal Egg Counts

The presence of gut nematodes and some approximate estimation of their number is usually inferred from finding, identifying and counting eggs in the faeces of the host. Three nematodes are known to infect possums in New Zealand, the eggs can readily be distinguished from one another by size and appearance, see Table 2.1. Possum faeces were collected from trays placed underneath the cages; while infections were being established these trays were washed with disinfectant [Savlon®] and placed underneath the cages for a 24hr collection period only. After initial stages of the trial it was considered sufficient for cage floors and trays to be washed thoroughly with water every 48hr.

The modified McMaster technique is used as the standard method of faecal egg counting in our laboratory [Manual of veterinary parasitological laboratory techniques, 1973]. This involves homogenizing 4gm of fresh [<24 hr] faeces with 50-60 ml of saturated salt solution, sampling from the centre of the stirred sample and counting eggs in an aliquot using a modified McMaster counting chamber [Whitlock, 1948].

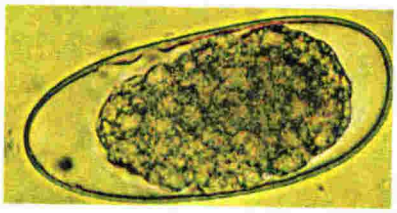
2.1.3] Development of a pure culture of *P. trichosuri*

There are few areas of New Zealand where *P. trichosuri* exists on its own without accompanying *Paraustrostrongylus trichosuri* or *Trichostrongylus* species [Heath *et al.*, 1997] but because of the method of infection of its host, a monospecific infection can readily be set up non-surgically for this parasite. A larval culture from a naturally infected Upper Hutt possum was set up as described below and the larvae harvested. About 10,000 mixed L3 larvae in 1-2ml of water were applied to the shaved abdomen of anaesthetized drenched possums which were then kept on their backs for 30min. After this time, which was deemed to be sufficient for the *P. trichosuri* larvae to penetrate the skin, the abdomen was washed carefully to prevent the possum infecting itself orally with other species. These possums developed monospecific *P. trichosuri* infections after about 14 days and served as a source of *P. trichosuri* eggs for larval culture or of parasitic adults.

2.1.4] Culture of L3 Filariform Larvae

Faeces were collected from possums that had been monospecifically infected with *P. trichosuri* and showed positive faecal egg counts. Two methods of obtaining L3 filariform

Table 2.1 The appearance and size of the eggs of the gastro-intestinal nematodes infecting the possum *Trichosurus vulpecula* in New Zealand

	Width of egg µm	*Length of egg* µm	*Description*
Parastrongyloides trichosuri 	29 [29-30]	58 [50-61]	**Clear shelled Usually embryonated when passed in faeces Very distinctive pencil-drawn appearance**
Paraustrongylus trichosuri 	40 [39-45]	80 [75-85]	**Rounded ellipse More defined membrane Blastomeres seen but no embryo**
Trichostrongylus colubriformis 	45 [41-50]	92 [84-109]	**Irregular ellipse Not very wide poles Smooth surface Not embryonated Blastomeres seen**

larvae were used depending on their intended eventual use. In the bulk production method when very large amounts of larvae were required for protein preparation, all the faeces available from infected animals were pooled, mixed by hand with equal volumes of vermiculite, kept moist, stirred to aerate daily and incubated at room temperature [19-23°C] for 10 to 14 days. The larvae, which resulted after several free-living cycles, were harvested by allowing them to migrate from the faecal/vermiculite mass through coarse then fine filters into distilled water; they were then cleaned by allowing them to migrate through a 22μm sieve into distilled water.

An alternative method of obtaining larvae at a more uniform stage of development was used when they were required for possum infection. Faeces from one infected animal were mixed with an equal volume of vermiculite and incubated at room temperature as shown in the diagram, Fig 2.1. Again, usually after 10 to 14 days, infective larvae produced in the faecal/vermiculite mass migrated down the wet filter paper slope into the distilled water and were found sedimented on the bottom or seen as cream-pinkish mass in the corners of the tray. The water plus larvae was collected and the larvae allowed to settle, centrifuged at low speed, approximately 500g, for 2 to 5 minutes, washed with distilled water, then used either for antigen preparation or to infect possums. For the latter, the larvae were uniformly re-suspended in distilled water then 100μl of suspension was added to 1ml of water on a counting slide and the larvae counted; the % viability was also calculated. The concentration of larvae was adjusted to give the desired dose in a volume of 2ml for transdermal infection or 1ml for infection by sub-cutaneous injection.

Larvae for antigen were prepared by centrifuging at 15,000g in a bench top centrifuge, to remove as much water as possible then re-suspended in a minimal volume of phosphate buffered saline [PBS]. A serine protease inhibitor, Pefabloc [Roche] was added at the manufacturer's recommended concentration [50μg/ml] to those aliquots of larvae that were to be used for protein isolation but not to those destined for DNA preparation. They were then frozen, thawed and thoroughly ground under liquid nitrogen in a chilled mortar with pestle; 1 ml of ground material was collected in a minimum volume of PBS. Approximately 2 volumes of ground larval material was suspended in 1 volume of PBS; the

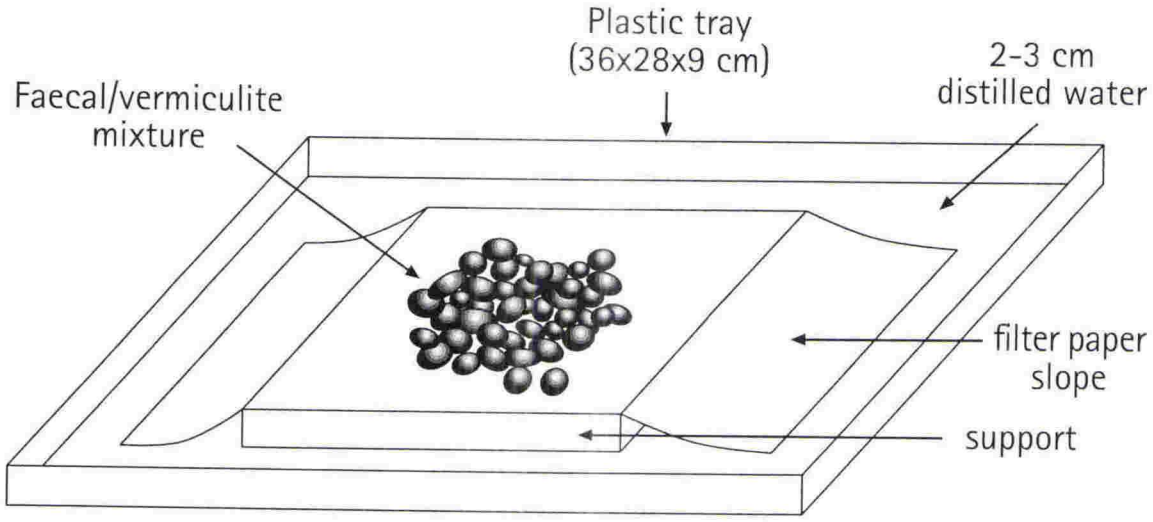


Fig 2.1 Culture of infective L3 larvae from *P. trichosuri*

Faeces from infected possums were mixed by hand with vermiculite at a ratio of 1:1. They were placed on wet filter paper on a support in a tray containing distilled water. Faeces were incubated at room temperature for 10 -14 days.

suspension was centrifuged at 15,000g in a bench centrifuge at 4°C for 15min and the supernatant removed and frozen in aliquots at –80°C until used as "larval antigen".

2.1.5] Parasitic adults

Possums were mono-specifically infected with *P. trichosuri*; after about 14 days standard faecal egg counts were done and counts of greater than 500 eggs per gram [e.p.g] indicated that *P. trichosuri* parasitic adults had established in the small intestine of the animal in sufficient number to be harvested. Those animals with faecal egg counts below 500 e.p.g. were not used for collection of adults. Animals were euthanased, the abdomen opened and the small intestine clamped at the pyloric sphincter and the ileo-caecal junction. The entire small intestine was dissected out, slit open longitudinally and the contents and mucosal layer scraped out. The contents were rinsed into a large screw topped jar with about 2 L PBS, then shaken and incubated at 37°C for 20-30 min. The top 60-75% of the contents was removed by suction, washed again with PBS then an aliquot of diluted contents transferred into a glass petrie dish where, using a dissecting microscope at low power, individual worms were removed into a clean dish containing PBS or half and half PBS/ Adult parasite culture medium [Appendix 3]. Individual worms were washed several times; if used for culture, they were transferred to Adult culture medium and kept at 37°C and 5%CO_2 under sterile conditions. Worms used for adult antigen were kept in PBS for several hours then treated essentially as larvae; a protease inhibitor [Pefabloc] was added, they were centrifuged, ground under liquid nitrogen, centrifuged and the supernatant was frozen.

2.2] Detection of the parasite antigen.

2.2.1Preparation of antibodies

Positive control serum: Previously, three New Zealand white rabbits were each injected subcutaneously and intramuscularly with 1ml of whole *P. trichosuri* larvae ground under liquid nitrogen at 1:1 in STM adjuvant [Bokhout *et al.*, 1987]. Two injections were given one month apart on contra-lateral sides. After a further month the serum was tested for

antibodies by immunoblotting and, when positive, the rabbits were bled, euthanased and the pooled serum kept at –80°C. This was intended as a positive control and used as "Rabbit-anti-Pt" serum [Heath, unpublished].

Positive possum serum: Serum samples were selected from possums with moderate or heavy monospecific infections with *P. trichosuri*, as found in the survey of parasites in New Zealand possums [Possum Access Database, AgResearch]. Details of possum sera that were found to have antibodies to the 70kD proteins of interest and were subsequently used for immunoblotting are shown in Table 2.2. Some serum samples that had antibodies to the *P. trichosuri* antigen of interest in the original immunoblots [Fig 1.8] were also used. These sera were used at concentrations of 1:1000, 1:100, 1:40 or undiluted depending on the strength of the antibodies or experimental requirements.

Negative control serum: As there appeared to be no parasites found in any possums from Stewart Island [Stankiewicz *et al.*, 1997b], serum from at least one of these possums was included in each immunoblots as a negative control. Negative sera were used at similar concentrations to positive sera.

Affinity-purified antibodies: Three samples of sera that appeared to have high levels of antibodies, in comparison with others on initial immunoblots, were chosen. Antibodies were isolated from these sera by affinity purification from nitrocellulose according to the method of Beall and Mitchell [1986]. Briefly, in this procedure proteins in 400µl of larval antigen were separated by electrophoresis then transferred to nitrocellulose by Western blotting; the membrane was stained with Ponceau S stain for 5min and the position of the molecular weight markers carefully marked. The nitrocellulose was blocked in 5% Blotto in 10mM Tris-HCl, pH 8.0, 0.15M NaCl, 0.05% Tween [TNT] for 2hr and a strip from 60 to 90kD was excised. The selected serum was diluted 1:25 in mouse osmolality PBS [see Appendix 3], 20% foetal lamb serum, 0.05% Tween 20 and incubated with the antigen strip overnight at room temperature. Following antibody binding, the strips were washed three times in mPBS, T_{20} then for 30min in 0.1M boric acid, 0.5M NaCl pH 8.0 and again washed three times. Antibodies were eluted from the nitrocellulose with 0.1M glycine-HCl pH 2.6, 0.15M NaCl for 2-3 min. Immediately following elution the pH of the eluate was

Table 2.2 Details of Possum serum used in Immunoblots

Possum serum number	Location	Age [yr]	Sex	Wt [kg]	Parasites present		
					Pt	Pu	Tc
86	Wanganui	4	M	2.5	280	0	0
129	Wanganui	4	F	2.3	30	0	0
292	Orongorongo	4	M	2.4	0	0	0
2120	Stewart Island	7	F	4.2	0	0	0
CA62	Upper Hutt	nd	M	2.3	*	0	0

*Possum CA62 was experimentally infected with 6000 *P. trichosuri* L3s. Adult worms were present in the gut at necropsy but they were not counted.

adjusted to pH 7.2 with a pre-determined volume of 2M Tris-HCl, foetal lamb serum to 20% was added and these antibodies used as "affinity-purified antibodies" in some following procedures.

2.2.2 Separation and Immunoblotting of antigen

Ground larval antigen prepared as in 2.1.4 was centrifuged at either 15,000g in a bench centrifuge at room temperature for 15 min or at 120,000g at 4°C for 5min in an air driven ultra centrifuge [Beckman Airfuge™]. Sample loading buffer [Laemmli, 1970] was added to the supernatant at 1:1, the sample boiled for 5min then applied to a 5-25% acrylamide gradient gel [Hames and Rickwood, 1981] either in individual wells at differing concentrations or across the entire gel. The gels were run in Tris-glycine buffer at 160V for 16 hours. Antigen from parasitic adults was prepared in a similar way.

Separated protein was transferred to nitrocellulose by Western blotting at 50V for 1hr in bicarbonate buffer [Towbin *et al.*, 1979]. The nitrocellulose sheet was blocked [to prevent non-specific binding of proteins] in 5% skim milk powder in high salt [500mM] Tris buffered saline [Blotto], see Appendix 3, cut into 2mm strips and incubated in trays with possum serum at various concentrations for 4hr or overnight at room temperature. The antigen/antibody complex was bound by rabbit serum containing antibodies raised to the total immunoglobulin fraction of possum serum [Rabbit-anti-total-possum-Ig] [McCarthy, unpublished]. This second antibody was used at concentrations of 1:100 or 1:1000; all antibodies were diluted in Blotto. Visualization was with a commercial goat-anti-rabbit horseradish peroxidase conjugate [GAR-HRPO] [Cappel, ICN Pharmaceuticals] at a dilution of 1:1000, developed with 4-chloro-1-napthol or amino ethyl carbazole [AEC].

2.2.3 Antibody specificity

Serum from possums monospecifically infected with one of the three possum nematodes found in New Zealand, was used to probe larval and adult antigen from *P. trichosuri*. Antigen aliquots of 100µl were separated by electrophoresis, transferred to nitrocellulose then probed with serum from possums infected as follows:

1] *Trichostrongylus colubriformis* [Tc] - five possums from Banks Peninsula with infections of 100-1500 worms.

2] *Paraustrostrongylus trichosuri* [Pu] - two possums from Shannon and a further four naïve South Island possums that had received truncated *Paraustrostrongylus trichosuri* experimental infections.

3] *P. trichosuri* [Pt] - six possums from Northland and Kawau with natural infections; these are areas where no *T. colubriformis* or *Paraustrostrongylus trichosuri* are found.

4] No exposure to infection - four possums from Stewart Island.

2.3] Determination of secretory nature of antigenic protein

2.3.1 Investigation of the excretory/secretory products of *P. trichosuri*

Excretory/secretory products of L3 larvae were collected and analysed. Three 24 well sterile culture plates [Nunc] were coated with an irrelevant protein [blocked], a 1% sterile solution of fish gelatin then allowed to dry. Larvae were obtained as described in 2.1.4. It had been found that larvae survived and maintained viability at room temperature best in distilled water rather than tap water, normal saline, PBS or "nematode PBS" [data not shown]; nematode PBS is a proprietary mixture of salts used for long-term nematode larval maintenance. Therefore larval cultures were set up in distilled water with additives as shown in Table 2.3. In addition, approximately 25,000 L3's were kept in 200ml dH_2O with 1% glucose in a coated 500ml culture flask. All plates and flasks were kept at room temperature. Approximately 1.25ml of culture fluid was removed from wells and replenished at 24, 48 and 72 hr; this was filtered through a 0.2µm filter to remove any larvae that had been inadvertently collected, then frozen in a blocked universal container. All samples were freeze dried then reconstituted in 1ml PBS. Samples were run on 5-25% acrylamide gels and duplicate gels were immunoblotted with positive serum as described in section 1. Larval cultures were repeated in sterile non-coated glass at a concentration of 5000/ml in dH_2O with 1% glucose, no other additives were used. Culture fluid was collected in sterile uncoated glass at 24 and 84hr, freeze dried and examined for immunogenic protein as before.

Table 2.3 ***P. trichosuri*** **L3 larval cultures for the collection of excretory/secretory products.**

Plate number	Well number	Number of larvae per well	Additives
1	A 1-6	10,000	1% glucose
	B 1-6	10,000	1% glucose
	C 1-6	10,000	0.1% glucose
	D 1-6	10,000	0.1% glucose + 1% * 'nematode PBS'
2	As above	5,000	As above
3	As above	25,000	As above

Final volume in all wells was 2 mls

* nematode PBS is a proprietary mixture of salts used for long term nematode larval maintenance (AgResearch)

Adult worms were removed from possum guts as described in 2.1.5. Two 24 well plates were blocked as before in 1% sterile fish gelatin and adults were added to the wells at: Plate 1- approximately 100 very clean worms per well. Plate 2- 50 worms per well. All adults were cultured in 2 ml of Adult culture medium without Nystatin, see Appendix 3. About 1ml of culture medium was removed from each well into siliconized vacutainers after about 60hr.

2.3.2 Immunohistochemistry of whole live *P. trichosuri* larvae and adults

Immunohistochemistry of whole live *P. trichosuri* larvae was attempted by directly reacting affinity purified antibodies with live animals and looking for evidence of an antigen-antibody reaction on the tegument. Cultures were set up with consideration given to ensuring that a true comparison could be made between a reaction of the specific antibody on the surface of the larva and non specific binding of any of the proteins of the detection system or other proteins secreted by the larvae.

Cultures were set up as in Table 2.5. A 96 well culture plate was blocked with sterile 1% fish gelatine; larvae were washed and approximately 200-300 were added in 0.1ml of distilled water. Reagents were added as shown in Table 2.5. Larvae were first coated with gelatine at 0.05% final concentration, the plate was agitated on an IKA-VIBRAX VXR at 300rpm for 30min; coating solution was removed and worms washed by adding 200µl distilled water and agitating for 5-10 min, twice. 100µl of affinity purified antibodies was added to the appropriate wells and the larvae incubated with shaking for 1-1.5hr, then washed five times with mouse tonicity PBS. Second antibody, Rabbit-anti-total-possum-Ig at 1:100, with foetal calf serum [FCS] at 1:100, was incubated with the larvae for 1-1.5hr and they were washed five times in mPBS. The third antibody, GAR-HRP or goat-anti-rabbit conjugated to fluorescein isothiocyanate [GAR-FITC] at 1:250 and 1:100 respectively was incubated for 1hr and then removed by washing five times again. Filtered AEC was add to all wells except where GAR-FITC had been used as third antibody and washed off with distilled water after 10min. All cultures were carefully inspected on an inverted microscope under low power and scored for the presence of clumps of protein of a darker colour than the background. Their appearance was compared with the control worms. Two observers independently scored the larvae at the time of colour development

and photographs were taken after 48hr when the camera became available. There was no UV camera available at the time to take photographs of the FITC labelled larvae.

Cultures were set up with adults [parasitic] instead of larvae under certain of the conditions, as shown in Table 2.5.

2.4] Isolation of antigenic protein

2.4.1 Preparative electrophoresis of larval antigen

In this method proteins are separated by electrophoresis through a vertical cylindrical resolving gel until they migrate off the bottom of the gel. Here they pass into an elution chamber where a dialysis membrane prevents protein leaking out into the re-circulating running buffer and a continuous flow of elution buffer carries them up out of the apparatus to a fraction collector. The apparatus, a BioRad Prep Cell Model 491, was set up according to the manufacturer's instructions; the experimental set-up is shown in diagrammatic form in Fig 2.2. Two full experiments were run. In the first, 20ml of 10% acrylamide was cast in a narrow column [28mm internal diameter] giving a 5.5cm resolving gel; in the second, 20ml of 7.5% acrylamide was cast. Gels were left to polymerize overnight at 4°C. In both cases a 4% stacking gel was cast on top of the resolving gel and allowed to polymerize for 2hr. The elution chamber was prepared by soaking frits in elution buffer under vacuum to remove trapped air then set up with buffer-soaked dialysis membrane placed between the support and elution frit. The gel assembly was then carefully removed from the casting stand and the whole apparatus assembled according to the operating manual.

In experiment one, 0.5ml of larval antigen, 0.5ml distilled water, 1.0ml X1 SDS loading buffer was loaded onto the top of the stacking gel. In experiment two, a sample with a total volume of 9ml was loaded; this comprised: 7ml larval antigen having a protein concentration of approximately 0.5mg/ml, 1.5ml X5 SDS loading buffer, 1ml 45kD coloured protein marker and 1ml 127kD coloured protein marker, the last two having been previously eluted from a PAGE gel. In both cases the sample was boiled for 5min, cooled then applied to the top of the stacking gel through a syringe with flexible Teflon tubing attached. The cooling and running buffer, Tris-glycine pH8.3, was re-circulated at between

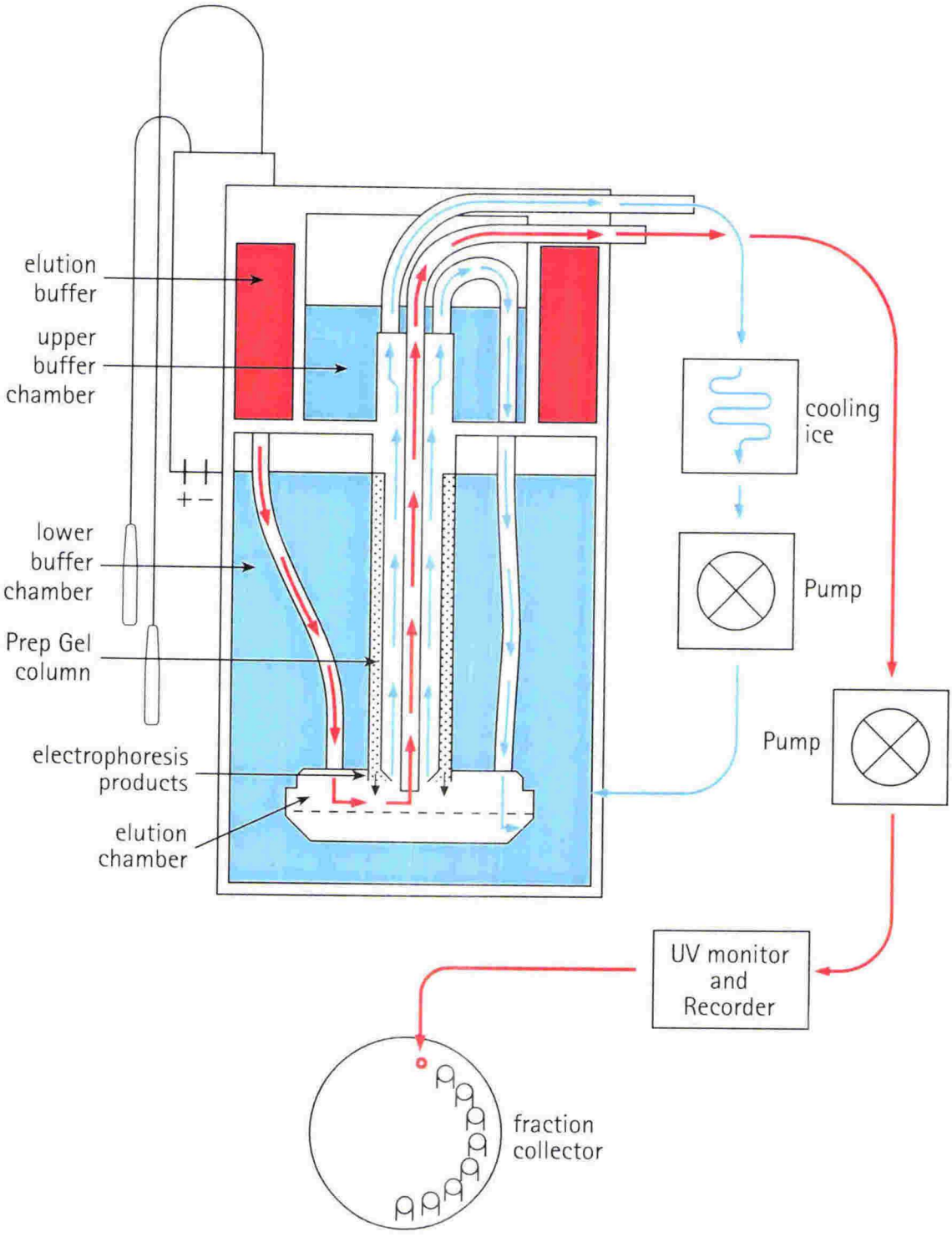


Fig 2.2 Experimental set-up of preperative gel electrophoresis using a Bio Rad Prep Cell

Pathway of cooling and electrophoresis buffer

Pathway of elution buffer plus samples

50 and 100ml/min with the outlet tube led through a container of ice and salt, as no refrigeration unit was available for this apparatus. The elution buffer, also Tris-glycine pH8.3, had a flow rate of 0.5ml/min during the collection periods and the majority of the run. This was replenished as needed.

A constant current of 40mA was applied; this gave an initial voltage of 140V which increased markedly over time. Eluted protein and buffer flowed through a UV monitor and fractions of 2.5ml were collected by a LBK fraction collector from the time the dye front migrated off the gel until it was believed that all the protein in the desired size range had migrated off the bottom of the gel. Experiment 1 continued for 21 hours, experiment 2 for 48 hours.

2.4.2 Protein identification

Four samples from experiment two were sent to the School of Biological Sciences, Auckland University [Auckland, New Zealand] for protein sequencing. Protein in Fraction 8 was diluted to reduce the concentration of SDS and purified then sequenced by Edman degradation.

Results:

2.2] Detection of the parasite antigen

2.2.1 and 2.2.2 Separation and immunoblotting of parasite protein

The 70kd protein that was the target of this investigation had been detected by electrophoresis of ground larvae and immunoblotting with serum from infected possums [Chapter I.5.3]. Nothing was known of its nature except its mass on electrophoresis and that it elicited an antibody response in possums. The detection system for this protein was re-established and the specificity of this protein to *P. trichosuri* was re-investigated, as a preliminary to attempting to isolate it.

Initial experiments showed that the crude larval and adult antigen consisted of a complex mix of proteins, see Fig 2.3. There appears to be a moderately prominent band at 70kD seen more clearly in Fig 2.4 this protein or proteins may correspond to the target antigen but it is not possible determine with surety on a one-dimensional gel. When separated larval antigen was probed with possum serum a considerable number of antibodies reacted with parasite proteins, see Fig 2.5. There is a protein at 94kD that is recognized by the serum of almost all possums; it remains at present unidentified. There are antibodies in infected possum serum that react with proteins of 68-78kD; results from two infected possums are shown in lanes 1-4, Fig 2.5. The reaction is not consistent, one two or three bands in this region can be seen in serum from possums with similar infections, see Fig 1.8, and, on occasions, no bands at all from laboratory possums that have had two infections with *P. trichosuri* and who would be expected to react [not shown]. Negative control serum, however, has never given a reaction in any of the numerous blots that have been done.

The reaction between antibodies that were affinity purified from serum 86 and antigen can be seen in Fig 2.6, lanes 3 and 6. The affinity-purified antibodies were weaker than the antibodies in serum and, in this blot, have been developed with a more sensitive colour system, AEC is about ten times more sensitive than the system developing the serum [4-chloro-1-naphthol]. It was hoped that these affinity-purified antibodies would be

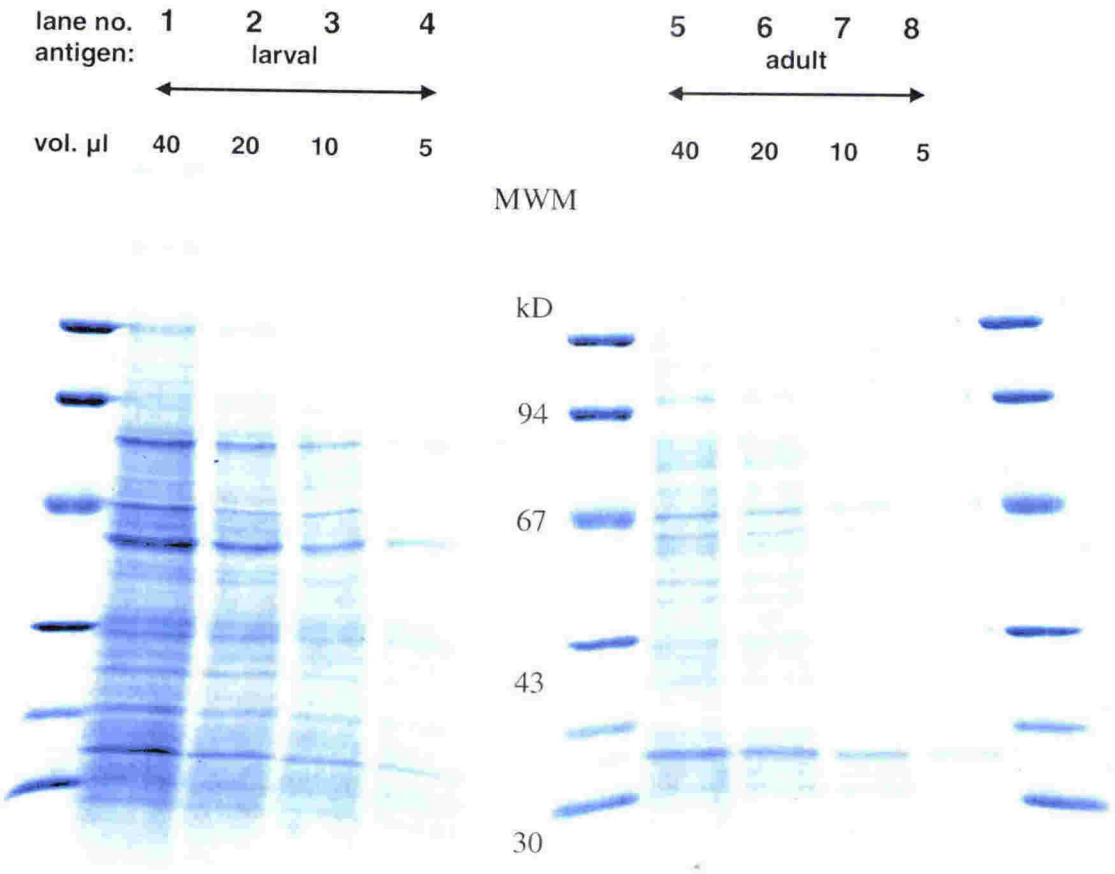


Fig 2.3 Polyacrylamide gel electrophoresis of *Parastrongyloides trichosuri* [Pt] larval and adult antigen.

Larval antigen was prepared by grinding Pt L3 larvae under liquid nitrogen and collecting the ground material in PBS at approximately 2:1v/v larvae/PBS. The supernatant from centrifugation of the extract at 15,000g for 15min was used for electrophoresis in lane 1-4 in decreasing amounts as shown.
Parasitic adults were prepared in a similar manner to larvae and the adult antigen was used in lanes5-8 in decreasing amounts as shown.
Protein was applied to a 5-25% gradient gel and run at 140V for 15hr.

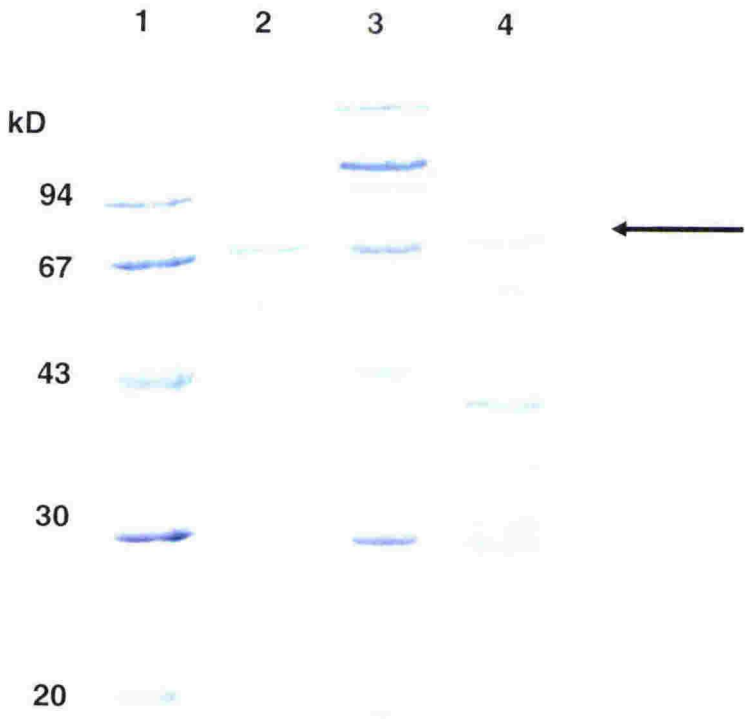


Fig 2.4 Polyacrylamide gel electrophoresis of *P. trichosuri* [Pt] larval and adult antigen showing the limit of resolution of proteins on a 1D gel. Different aliquots of larval and adult antigen have been used, compared with Fig 2.3, showing variation in protein profiles between aliquots of antigen.

Larval antigen was prepared by grinding Pt L3 larvae under liquid nitrogen and collecting the ground material in PBS at approximately 2:1v/v larvae/PBS. 20µl of supernatant from centrifugation of the extract at 15,000g for 15min was used for electrophoresis in lane 2.
Parasitic adults were prepared in a similar manner to larvae and 20µl ofthe adult antigen was used in lane 4.
Lane 1- low molecular weight markers; lane 3- broad range molecular weight markers
Proteins were applied to a 7.5% acrylamide gel and run at 140V for 2hr. 200V for 4.5hr

⟶ Indicates position of protein to which characteristic antibodies are produced by possums infected with *P. trichosuri* but not other possum parasites.

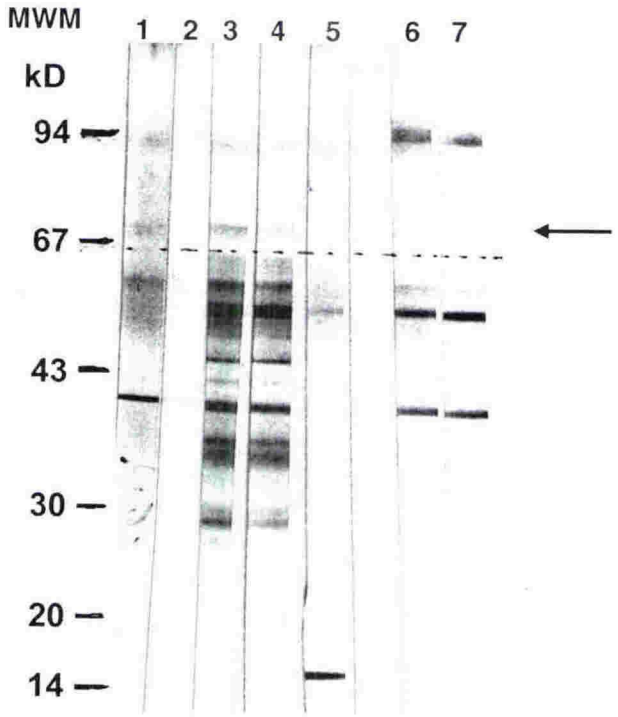


Fig 2.5 Immunoblot of *P.trichosuri* L3 antigen reacted with infected possum serum

Larval extract was electrophoresed on a 5-25% acrylamide gel and the proteins were transferred to nitrocellulose. Nitrocellulose strips were reacted with infected possum serum from two different possums at two different concentrations: lane 1 – 292 at 1:100, lane 2 – 292 at 1:1000, lane 3 – 129 at 1:100, lane 4 - 129 at 1:1000.Lane 5 was reacted with negative control serum, from possum 2120 at 1:100 and lanes 6, 7 were reacted with positive control serum: lane 6 - Rabbit-anti-Pt serum at 1:100 lane 7 –at 1:1000,

⟶ Indicates the position of the characteristic antibodies, to a component of larval antigen, that are seen in possum serum from possums infected with *P. trichosuri* but not with other possum parasites.

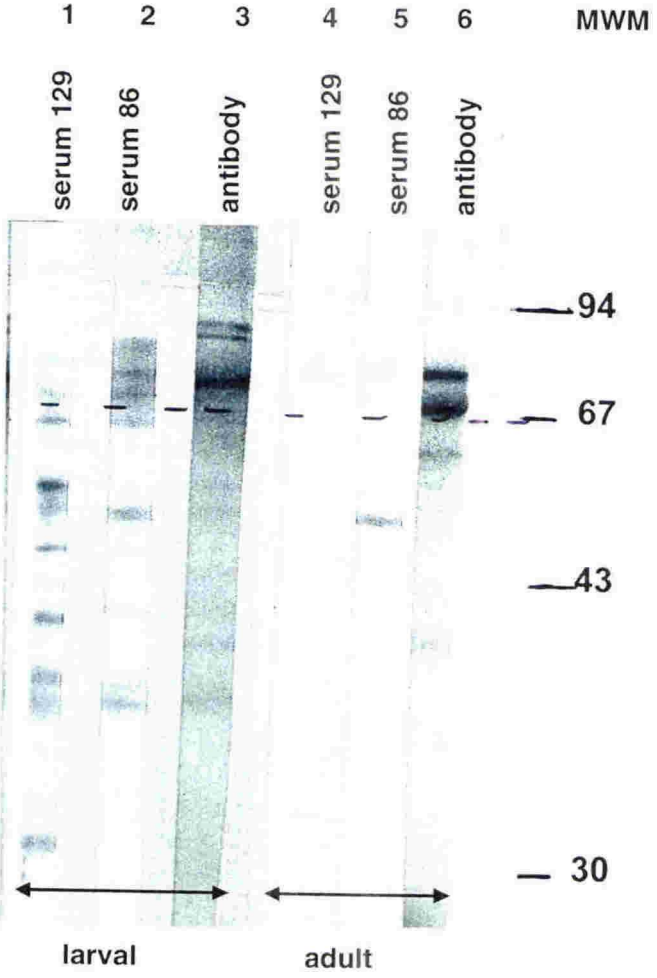


Fig 2.6 Immunoblot of reaction of serum and of affinity-purified antibodies with *P. trichosuri* antigen .

Larval extract was electrophoresed on a 5-25% acrylamide gel and the proteins were transferred to nitrocellulose. Lanes 1-3 are nitrocellulose strips from the Western blot of larval antigen. The nitrocellulose strips were reacted with: lane 1 -serum 129 at 1:100, lane 2, –serum 86 at 1:100, lane 3, –affinity-purified antibodies from serum 86 at 1:50.
The procedure was repeated with adult extract, lanes 4-6 are nitrocellulose strips from the Western blot of adult antigen. The nitrocellulose strips were reacted with: lane 4 -serum 129 at 1:100, lane 5, –serum 86 at 1:100, lane 6, –affinity-purified antibodies from serum 86 at 1:50.
Reactions with two different colour substrates were used. Initially all strips were developed with 4-chloro-1-naphthol but there was no discernible reaction in lanes 3 and 6. Lanes 3 and 6 were re-developed with AEC which is 10X more sensitive than 4-chloro-1-naphthol.

sufficiently specific to pinpoint the target antigen protein as a single band. Fewer bands are visible when immunoblotting with serum but there are several, and the bands seen on the blots are still too diffuse to pinpoint with accuracy one band only from the many on an acrylamide gel.

Antibodies were purified from one of the most strongly positive sera and the reaction of serum and antibodies compared by blotting both larval and adult antigen. Fig 2.7 shows that there are a number of similar bands but the profiles are not identical. There does appear to be a band in the 67-75kD area of interest in the adult as well as the bands in this area in the larval antigen. This was surprising as the initial surmise was that this antigen was a protein involved in skin penetration whose presence, therefore, would be restricted to the infectious larval stage.

2.2.3 Antibody specificity

It was thought desirable to confirm that the presence of antibodies to the target protein was restricted to the serum of possums that were, or had been, infected with *P. trichosuri* only. Serum from possums where the species of infecting nematode could be known with certainty was used to probe larval and adult antigen and the results shown in Table 2.4. Bands were recorded as the blot developed. As expected there were no antibodies, corresponding to those on the previous immunoblots, detected in the serum of possums infected with *T. colubriformis* or with no nematodes [from Stewart Island]. Three possums experimentally infected with *Paraustrostrongylus trichosuri* showed no presence of these antibodies, one was indeterminate. Both the Shannon possums with natural *Paraustrostrongylus* infections but no current *P. trichosuri* infections showed the presence of antibodies. *P. trichosuri* is endemic in this area and it would seem likely that they had been infected previously. Four of the possums with natural *P. trichosuri* infections had antibodies in their serum, two did not, again showing that the presence of adult worms in the intestine of a possum does not always correlate with antibodies in the blood.

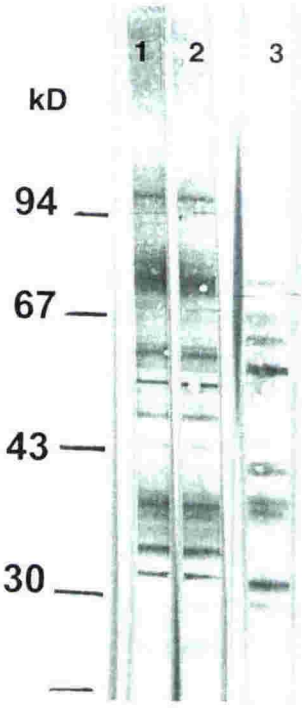


Fig 2.7 Immunoblot of reaction of *P. trichosuri* larval and parasitic adult antigen with serum from a possum positive for antibodies to an immunogenic parasite protein of 70kD

Larval and adult extract was electrophoresed on a 5-25% acrylamide gel and the proteins were transferred to nitrocellulose. Lanes 1 and 2 are nitrocellulose strips from the Western blot of adult antigen, lane 3 is from the Western blot of larval antigen. The nitrocellulose strips were reacted with possum serum 129 at 1:100 The Immunoblot was developed with AEC.

Table 2.4 Antibody Specificity - Immuno blot results

Location	Serum no.	Age (yrs)	Sex	1° Infecting Parasite number of worms			2° Bands present	
				Pt	Pu	Tc	Larval Ag	Adult Ag
Banks Peninsula	798	1	M	-	-	700	-	-
	714	4	M	-	-	1200	-	-
	737	1	F	-	-	100	-	-
	754	4	F	-	-	100	-	-
	789	4		-	-	1500	-	-
Shannon	1307	1	M		30		+	+
	1242	6	F		210		+	+
Lewis Pass Experimental Infections	96FSBAT	nd		-	Worms present	-	-	-
	F8CFBBT	nd		-	Not counted	-	-	-
	62A85TT	nd		-	Not counted	-	-	-
	62FBFBT	nd		-	285	-	+/-	-
Northland	3214	0	M	310	-	30	-	+/-
	3219	4	M	110	-	150	+	+/-
Kawau	695	7	M	200	-	-	+	+/-
	556	18	F	80	-	-	+	+/-
	555	2	F	50		-	+	+/-
	582	1	M	30		-	-	+/-
Stewart Island	2113	6	M	-	-	-	-	-
	2118	4	F	-	-	-	-	-
	2119	0	M	-	-	-	-	-
	2088	2	F	-	-	-	-	-
Positive control serum				-	-	-	+	+
Negative control no serum							-	-

*1 Infecting Parasite
Tc = *Trichostrongylus colubriformis* , Pu = *Paraustrostrongylus trichosuri* , Pt = *Parastrongyloides trichosuri*

2* Bands Present = positive reaction bands between 67 and 75 kD present. These were read when blot was first developed.
Ag = antigen
\+ = one, two or three positive bands seen, faint or strong
+/- = very faint, possible bands seen

2.3] Determination of secretory nature of antigenic protein

2.3.1 Investigation of the excretory/secretory products of *P. trichosuri*

Initially it was hypothesised that the 70 kD target protein was most likely secreted by the L3 infective larvae as they penetrate the skin of the host thus exposing the protein to the immune system of the host which then mounted an antibody response. This hypothesis was investigated by attempting to find this protein in the excretory/secretory [E/S] products of L3 larvae and in the E/S products of parasitic adults also. The filariform larvae had not been stimulated by contact with possum skin or exposure to higher temperatures and there were considerable difficulties in culturing adults in any conditions that would approximate the possum small intestine but despite these limitations, the parasites survived and protein was collected from the culture fluid. The target antigenic protein was looked for on the surface of the live parasites also.

It had been found that infective larvae survived well in distilled water for up to three weeks at room temperature with only slowly decreasing viability. As long as there was a wide surface area exposed to the air, shaking or rolling was not necessary for their survival, therefore, leaving the larvae in water with a simple energy source was an adequate culture method to obtain quite large amounts of protein [as in Fig 2.8]. It was necessary to prevent secreted proteins adhering to the plastic of the culture dishes or collection containers and so, at the outset, these were blocked with an irrelevant protein, sterile fish gelatin. When it was seen that larvae survived and seemed equally viable [as judged by movement] in all wells regardless of additive, culture medium from all wells was pooled. There was therefore about 30ml of liquid per sample before lyophilization.

The results of electrophoresis on the lyophilized samples showed faint double bands at 45kD in all samples and some very faint lower molecular weight bands. There was nothing visible at higher molecular weights but a high background smear, Fig 2.8. A number of areas of the immunoblots appeared to react with positive serum but, except for some of the low molecular weight bands, the results were too diffuse to give useful information, see Fig 2.9. It was thought that the blocking protein could be masking proteins in the E/S so larvae were cultured in unblocked glass containers. The culture fluid from the second larval

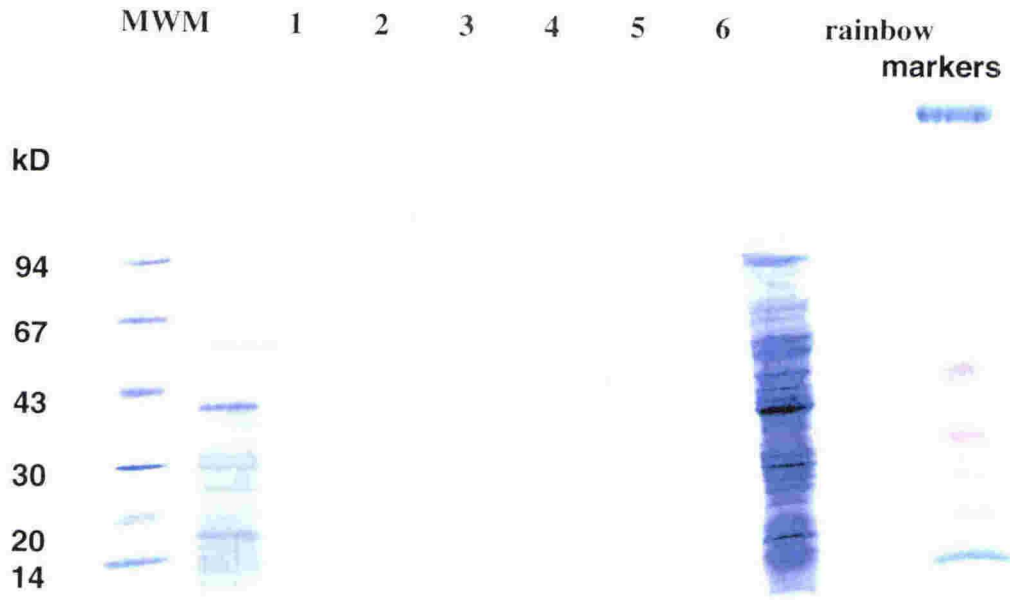


Fig 2.8 Electrophoresis of excretory/secretory products of *P. trichosuri* [Pt]L3 larvae.

P. trichosuri L3 larvae were cultured in distilled water with 1% glucose at different larval densities. The culture vessels were blocked with a solution of 1% fish gelatine. The excretory/secretory [E/S] products were collected after 24hr, lyophilized and reconstituted in 1ml PBS. The E/S products were electrophoresed on a 5-25% acrylamide gradient gel; the volume of protein loaded in lanes 2-5 is 60µl.

Lane 2 contains E/S products from the culture plate with larval density at 10,000 larvae/ml, lane 3 products from the plate with larval density at 5000 larvae/ml and lane 4and 5 E/S products from the pooled contents of culture flasks having a larval density of 5,000 larvae/ml.

Aliquots of two different Pt larval extract were run for comparison in lanes 1 and 6; volumes of 25µl were loaded on the gel.

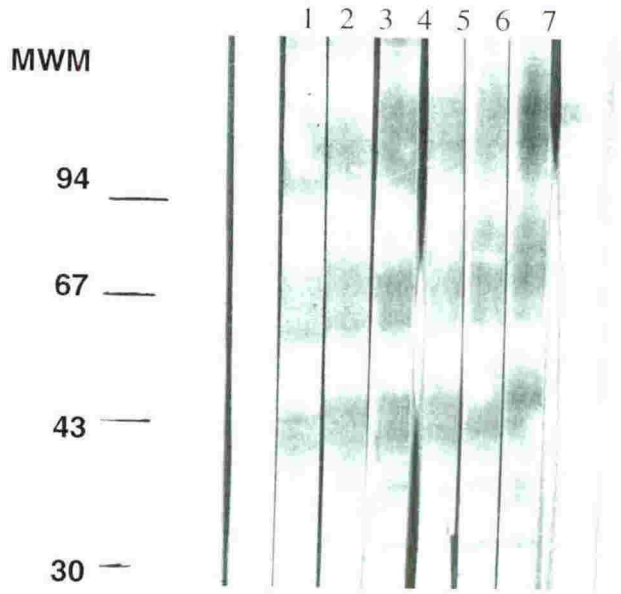


Fig 2.9 Immunoblot of excretory/secretory products of *P. trichosuri* [Pt] L3 larval culture with infected possum serum.

P. trichosuri L3 larvae were cultured in distilled water with 1% glucose at different larval densities. The culture vessels were blocked with a solution of 1% fish gelatine. The excretory/secretory [E/S] products were collected after 24hr, lyophilized and reconstituted in 1ml PBS. The E/S products were electrophoresed on a 5-25% acrylamide gradient gel, transferred to nitrocellulose and nitrocellulose strips were blotted with possum serum CA62 at 1:100.
Lanes 1 and 2 contain E/S products from the culture plate having a larval density of 10,000 larvae/ml, lanes 3 and 4, a larval density at 5000 larvae/ml and lanes 5 and 6 from culture flasks with a larval density of 5000 larvae/ml. Lane 7 is a blot of a nitrocellulose strip of Pt larval antigen from lane 1 in Fig 2.8.

culture in glass showed a whole spectrum of protein bands rather than a smear but no conclusive bands were seen on the immunoblots [not shown]. Culture medium from adult cultures showed a great deal of protein but the immunoblots were negative [results not shown].

2.3.2 Immunohistochemistry of whole live *P. trichosuri* larvae and adults

Attempts were made to see if the protein of interest could be detected on whole live parasites by reacting them with affinity-purified antibodies, then detecting the reaction with rabbit-anti-possum-total-Ig then goat-anti-rabbit conjugated to either HRPO or FITC. The appearance of larvae that had reacted with antibody is shown in Fig 2.10. The results of scoring the different cultures are shown in Table 2.6. These qualitative results showed considerable variation between replicate cultures; this could be due to a number of factors, one possible factor being the number of larvae that had died and degenerated during the experiment.

Some information can, however, be gathered. Under the culture conditions the antibody and detection system reacted with the ground larval antigen, which therefore acted as a positive control; the results are shown in Table 2.6, culture no. 9. Culture no. 7 acted as the negative control containing blocked larvae only. There is evidence that the affinity-purified antibody reacted with the antigen on the larval surface with the HRPO system [cultures no. 2 and 3]. Cultures no 4, 5 and 6 are controls in which antibody1, antibody 1 and 2 or antibody 2 and 3, are missing. When these are compared with the test cultures [2 and 3] it would seem that the second and third antibody also bind to the larvae although to a lesser extent. Cultures no. 10 and 11 using the FITC system were observed under UV light; the observations in this case were clear-cut. Larvae that had reacted with antibody displayed fluorescence in a kind of broken camouflage pattern on parts of the tegument compared with a pale uniform background fluorescence; no fluorescence seen on the larvae themselves in the negative controls [no photographs available].

Coating wells and larvae with a blocking protein was necessary and gave more consistent results, as comparison of culture no. 1, unblocked, with cultures no. 2 and 3 showed, also in the comparison of culture no. 12 with 13. Possum albumin was used to compare a species-

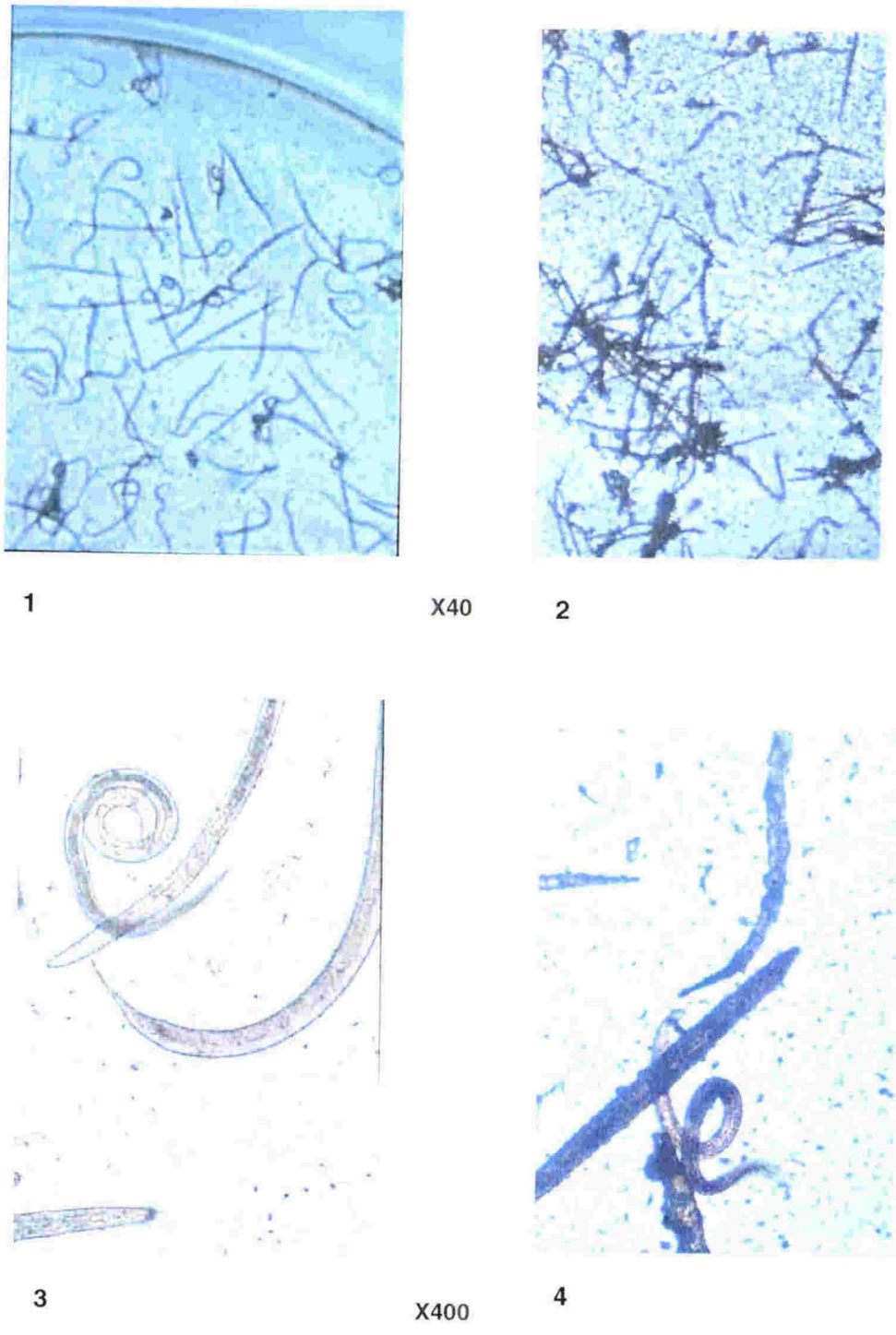


Fig 2.10 Immunohistochemistry of whole live *P trichosuri* larvae

Larvae were blocked in 1% fish gelatine then reacted with affinity-purified antibodies from possum serum. Pictures 1 and 3 are of negative control larvae in which no affinity-purified antibody was added. Pictures 2 and 4 are of larvae which have reacted with antibodies.

Table 2.5 **Immuno histochemistry of whole *P. trichosuri* larvae. - Experimental Conditions**

Reagents	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Blocking gel	-	✓	✓	✓	✓	✓	✓	✓	-	✓	✓	✓	-	✓	✓
Ab1	✓	✓	✓	-	-	✓	-	✓	✓	✓	-	-	-	✓	-
Ab2	✓	✓	✓	✓	-	-	-	✓	✓	✓	✓	✓	✓	✓	✓
Ab3 - GAR - HPRC	✓	✓	✓	✓	✓	-	-	✓	✓			✓	✓	✓	✓
Ab3 - GAR - FITC										✓	✓				
Pt larva	✓	✓	✓	✓	✓	✓	✓	-	-	✓	✓	✓	✓	-	-
Pt - crude Ag									✓						
Tc larva unsheathed														✓	✓
Possum Albm												✓	✓		
Adult cultures			✓	✓	✓					✓	✓				
Reasons for culture	Blocking	Test	Duplicate test	Negative control, specific Ab	Control, Ab3 only	Control of all detection proteins	Control, larvae only	Control, protein only no larvae	Positive control unblocked	Test FITC	Negative control, FITC	Ab specificity	Ab specificity	Test, species specificity	Negative control species specificity

Ab = antibody. Ab1 = affinity-purified antibodies from positive possum serum
Ab2 = Rabbit-anti-total-possum-Ig at 1:100
Cultures were set up in triplicate

Ab3 = Goat-anti-rabbit Ig conjugated to either HRPO or FITC
Possum Alb = possum albumin

Table 2.6 Immuno histochemistry of whole *P. trichosuri* larvae - Results.

Culture lane no.	Row A	Row B	Row C	Extra Observations	*Reason for culture
1	+	++	++	Big lumps in background and media	Effect of blocking
2	+++	++	+	*	Test, all conditions positive
3	++	++	+/-	*	Test, duplicate
4	++	+	+/-	*	Negative control, no Ab1
5	+	+/-	-		Control, Ab3 only
6	+	+	-		Control detection proteins.
7	-	-	+/-		Control, larvae only.
8	-	-	-	pale pink background	Control, protein only, no larvae.
9	+++	++++	++++	dark brown liquid and lumps = extensive	Positive control, unblocked.
10	+++	++	++		Test Ab3 - FITC.
11	-	-	-		Negative control FITC.
12	-	+/-	+		Comparison with possum albumen
13	+++	-	-	big lumps of dark brown debris	Duplicate unblocked
14	-	-	-		Species specificity - Tc larvae
15	-	-	-		Species specificity - Tc negative control

Key Score

+++ most larvae coated with several or many blobs of protein
++ many coated or some coated with many blobs
+/- some blobs on occasional worms or background
- nothing seen on larvae (some background on occasions).
* more than three cultures done results are averages.

specific protein with the antigen-specific system, see culture no. 12; there is a very slight reaction with blocked larvae and the albumin seemed to stick to uncoated larvae. The *P. trichosuri* antibody did not stick to the surface of *T. colubriformis* larvae at all.

The adult cultures were hurled from the shaker during final incubation. It proved impossible to save the worms and this work was not repeated

2.4] Isolation of antigenic proteins

A number of different attempts at dissecting out individual proteins around the 70kD size were made. Initially, one-dimensional SDS-PAGE on low 7.5 and 5% slab gels were run but it became clear that this would not provide sufficient discrimination and with gels below 5% the bands were too diffuse. An initial 2D gel was run; larval antigen was separated by two-dimensional electrophoresis using an Investigator 2-D Electrophoresis System [Millipore] apparatus. This showed that there were several proteins of a very similar molecular weight. There was one prominent dot at 70kD but also a cluster of four or five proteins in this size range with iso-electric points [pI] toward the lower end of the range, see Fig 2.11. At this time we had no ability to sequence proteins from the minute amount of material that could be eluted from the very small spots excised from gels such as this. Also the diffuse nature of the spots on immunoblots would have made a decision on which was, in fact, the immunogenic dot very difficult. It is interesting to compare this gel with that shown in Fig 5.10.

Many standard protein purification methods require a greater amount of protein than was initially available or knowledge of its chemical properties. Some preliminary purification was done by anion exchange separation using DEAE; initial small-scale experiments suggested that the pI of the immunogenic protein was below pH 7.0, possibly between pH 5-6. Finally, sufficient larval material was obtained to attempt preparative electrophoresis by SDS PAGE.

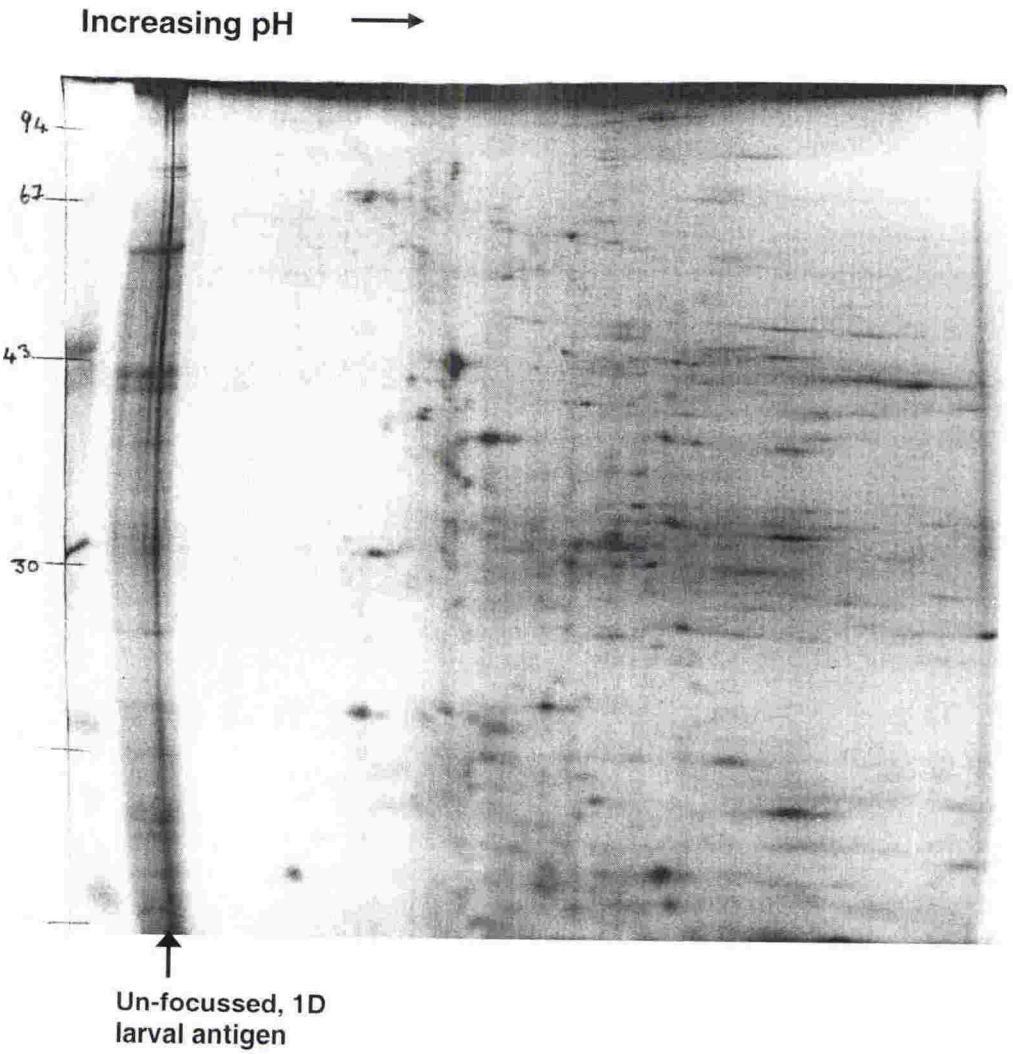
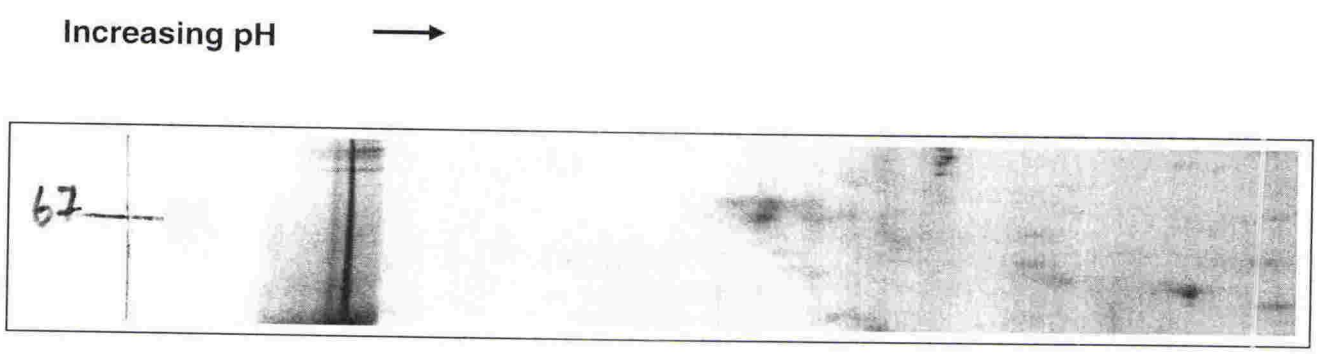


Fig 2.11 2D Polyacrylamide gel electrophoresis of *P. trichosuri* larval antigen. Approximately 80µg of total protein was subjected to isoelectric focussing in a wide range [pH 2-12] ampholyte. 2nd dimension electrophoresis was carried out on a 10% acrylamide gel.
The area of the gel around the 67kD marker is shown enlarged in the section below.



2.4.1 Preparative electrophoresis

In experiment 1, the bromophenol blue dye front in the loading buffer migrated off the gel after 2¼ hours and fractions were collected from that time continuously. Each fraction was 150 drops, about 2.5ml, and 200 fractions were collected then analysed. It proved difficult to correlate the peaks on the UV monitor with fractions that might have contained protein bands, so five tubes were collected over what was hoped to be a protein peak and these were pooled for analysis, rather than simply selecting samples at regular intervals. These were run in duplicate on gradient gels, Gel1 from experiment 1 is shown in Fig 2.12. Well separated bands of ascending molecular weight protein can be seen, however, no protein of over a molecular weight of 40kD was visible. . It would seem that the higher molecular weight proteins had either not passed through the 10% resolving gel for some unknown reason or that the electrophoresis had not continued for long enough although this latter was unlikely as collecting had continued for several hours after fraction 163 [lane 9, Fig 2.12] had migrated off the gel. The procedure was repeated using a lower concentration of acrylamide to ensure all proteins of interest were eluted from the gel column.

In experiment 2, preparative electrophoresis was repeated using a 7.5% resolving gel. Samples were collected from 19hr after starting the run till 48 hours had elapsed. The coloured marker proteins of 45 and 127kD were intended to bracket the proteins of 70kD thereby indicating when to collect them, but they gave no perceptible UV signal nor could the colour be seen on the gel itself. It was assumed that they were too dilute to be helpful

Pools of five tubes were concentrated using a Filtron [Microsep] 10kD or 30kD membrane then analysed. Analytical minigels run during the experiment indicated that the proteins of interest had come off the gel earlier than anticipated. Electrophoresis of the first samples, after concentration, can be seen in Fig 2.13, all proteins with a molecular weight lower than 65kD were in the pre-fractionated eluate, except for some possible contamination in fraction 19. Fig 2.14 shows proteins from 67-90kD. There appears to be a single predominant band of 70kD in lanes 2-5, fractions 4, 5, 6 and 8. Lanes 7 and 8, fraction 12 and 13, contain two proteins that are distinguishable on the gel; the lower band appears to be equivalent to the protein in lanes 2-5, the other may correlate with the slightly larger protein in lanes 9-11 taking into consideration the slight concave [downwards] distortion of

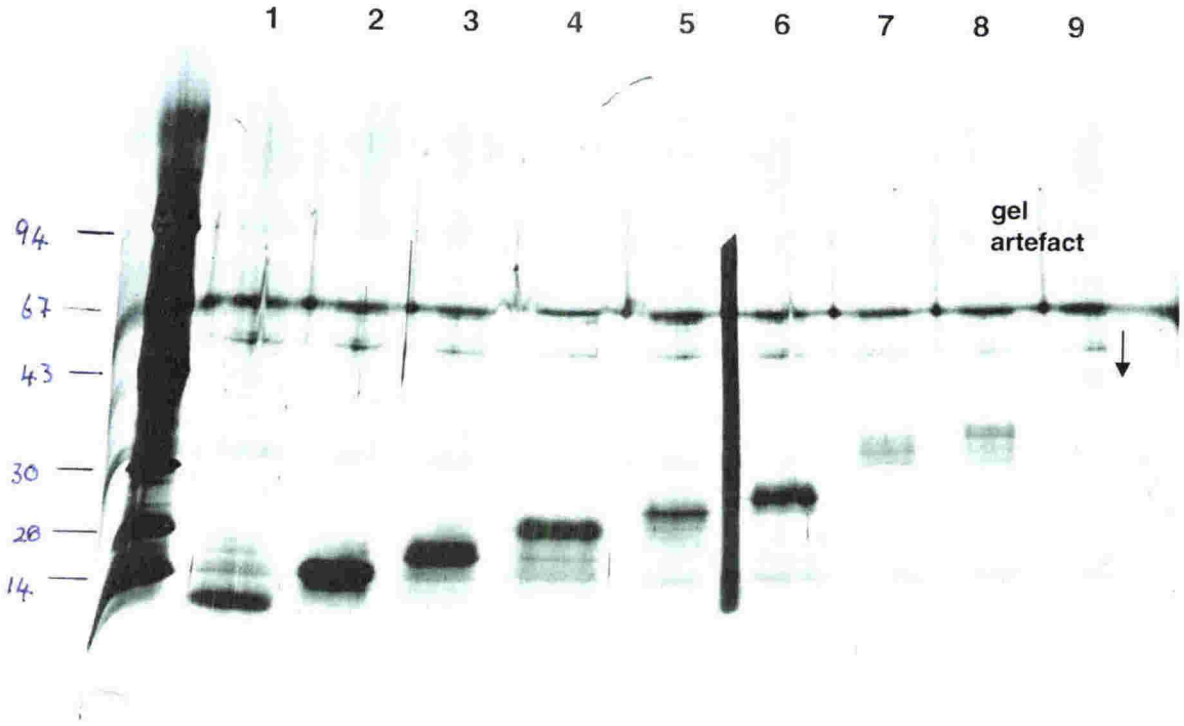


Fig 2.12 First preparative electrophoresis experiment. Electrophoresis of early samples obtained from the Prep Cell resolving gel column.

Proteins migrating off the bottom of the resolving gel column were collected in fractions of 2.5ml continuously from 2 to 21 hr from the start of the experiment. Five contiguous fractions were pooled and 75µl of unconcentrated eluate was electrophoresed on a 5-25% acrylamide gradient gel at140V for 15hr and then silver stained

Lane	Fractions
1	27→31
2	47→51
3	59→63
4	73→77
5	82→86
6	88→92
7	109→113
8	114→118
9	159→163

The band across whole gel at 70 kD is an artefact, possibly due to DTT concentration or to a sucrose irregularity while forming the gradient

k

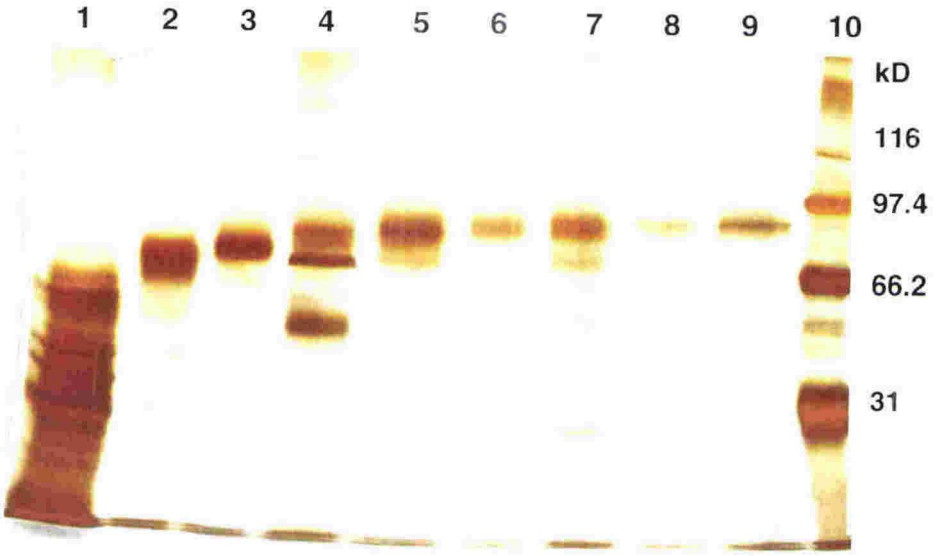


Fig 2.13 Second preparative electrophoresis experiment –analysis of early samples.

Initial fractions eluted off the Prep Cell resolving gel were concentrated by centrifugation through a Filtron Microsep 10kD cut off membrane then electrophoresed on a 10% analytical mini gel and silver stained. [Samples are individual fractions, not pooled].

Lane	Fraction
1	pre-collection
2	5
3	13
4	19
5	22
6	24
7	26
8	28
9	30
10	broad range molecular weight markers

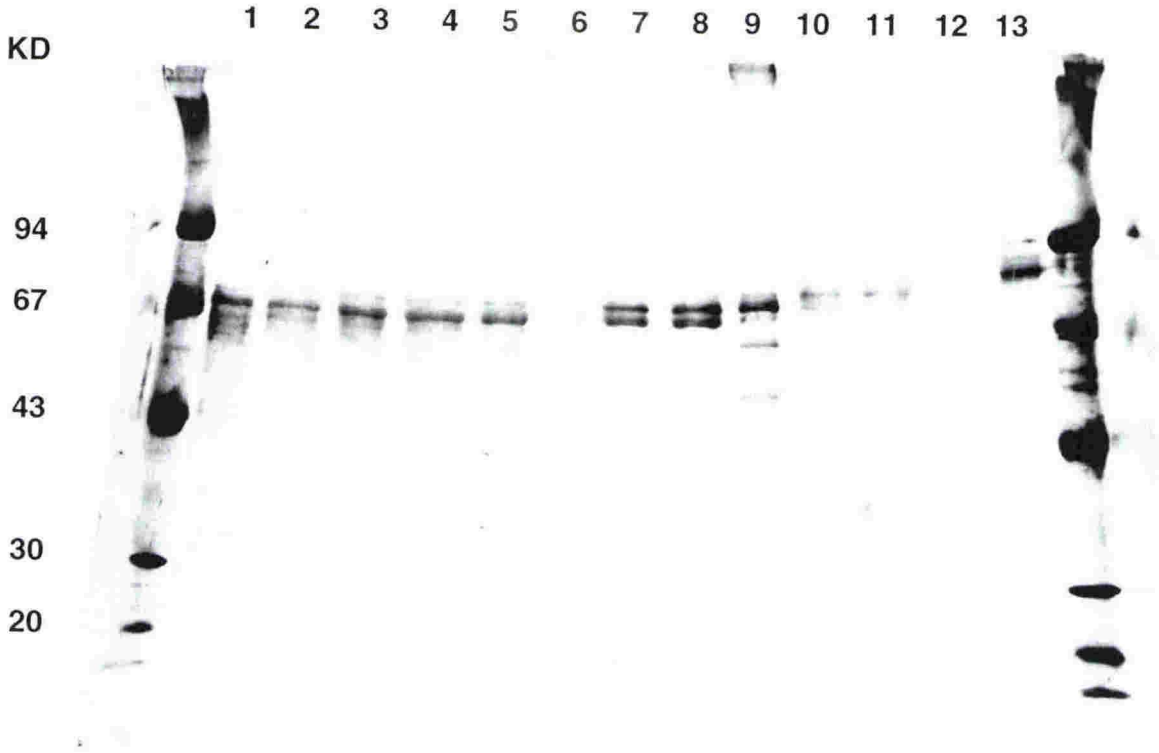


Fig 2.14 Second preparative electrophoresis experiment - Fractions containing proteins of MW 67-90kD.

Proteins migrating from the bottom of the Prep Cell resolving gel column were collected in fractions of 2.5ml. Individual fractions containing proteins with molecular weights of between 67 and 90kD were concentrated by centrifugation through a Filtron Microsep 10kD cut off membrane then electrophoresed on a 5-25% acrylamide gradient gel for 140V for 16hr and silver stained.

Lane	Fraction	Lane	Fraction
1	2	2	4
3	5	4	6
5	8	6	10
7	12	8	13
9	16	10	20
11	22	12	24
13	30		

the gel. In fraction 16, 20 and 22 the larger protein is present as a single band. A duplicate gel was transferred to nitrocellulose and probed with affinity-purified antibodies. From Fig 2.15 it can be seen that there was a discernible reaction with the antibodies in lanes 3-11. In lanes 3-7 fractions 5, 6 and 8 there is a single band; two proteins appear to have reacted with the antibodies in lane 8, fraction 13 and the slightly larger protein in lane 9, 10, 11, fractions 16, 20 and 22 also reacted with the antibodies. It appears that there are two immunologically reactive proteins of very nearly the same size in the larval antigen. Fraction 5, 6 and 8 appeared to contain one protein predominantly and this appeared sufficiently pure to sequence.

2.4.2 Protein identification

Protein in fraction 8, from the second preparative electrophoresis experiment [see Fig 2.14 and 2.15, lane 5], could be sequenced from the N terminal and nine amino acids of sequence were obtained: (A)-K-A-N-A-I-G-I-D-L-. There was a high background level of amino acids for all residues that was not due to contamination with buffer. There was a slight possibility that the sample contained another component, possibly a blocked protein; this did not discredit the quality of the sequence obtained for the major protein. Fractions 13 and 14 were also sent for sequencing in order to attempt to identify the second protein but no sequence information was obtained from these.

When a BLAST search was performed the nine amino acid sequence showed homology to the N terminal sequence of the Hsp70 proteins. There are three signature patterns for the Hsp70 family of proteins [Prosite PS00297, PS00329, PS01036], the first centres on a conserved N terminal pentapeptide; almost all of the large number of proteins known to belong to this class have this consensus sequence: [IV]-D-L-G-T-[ST]-x-[SC] and no other proteins in the database are detected by this pattern.

		consensus sequence	
Prosite signature 1	A-N-A-I-G-	I-D-L-G-T-T-Y-S-	C-V-G-V-F
Derived amino acid sequence	K-A-N-A-I-G-	I-D-L-	

It appears highly likely that the immunogenic protein of 70kD derived from *P. trichosuri* infective larvae is a heat shock 70 protein.

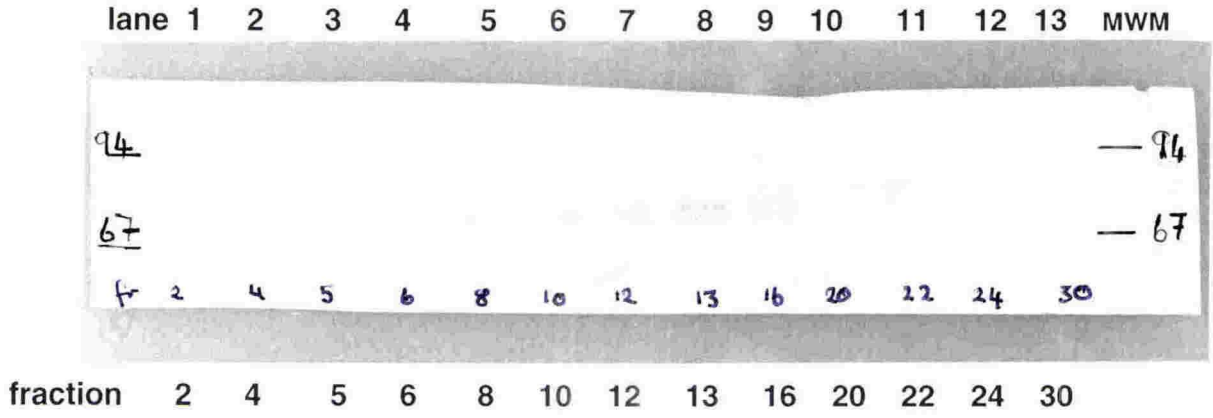


Fig 2.15 Immunoblot of fractions containing proteins of MW 67-90kD from the second preparative electrophoresis experiment.

Individual fractions containing proteins with molecular weights of between 67 and 90kD were concentrated by centrifugation through a Filtron Microsep 10kD cut off membrane then electrophoresed on a 5-25% acrylamide gradient. The gel was transferred to nitrocellulose which was then blotted with affinity purified antibodies from sera 129 and 86 [pooled] at a dilution of1:4.

Lane	Fraction	Lane	Fraction
1	2	2	4
3	5	4	6
5	8	6	10
7	12	8	13
9	16	10	20
11	22	12	24
13	30		

Discussion

Little is known at present about the nature of the possum's response to infection with *P. trichosuri*. There is evidence that resistance to infection may develop naturally; conversely, possums in Kahurangi National Park, New Zealand, can lose primary infections and then become re-infected within one year [Ralston, personal communication]. Resistance to challenge did develop to some extent after experimentally induced infections were truncated with anthelminthics [Heath *et al.*, 1999] and there was a correlation between the presence of antibodies recognizing the 67-80 kD region of larval antigen and resistance to challenge infection. The presence and number of adult worms after challenge does not so far correlate with the level of antibody observed on immunoblots but further work to show a relationship between antibody levels and the presence or absence or intensity of infection may be done in the near future.

In the North Island, as has been stated, *P. trichosuri* is ubiquitous and about 80% of possums become infected in their first year of life [Heath *et al.*, 1997] so it is reasonable to assume that almost every North Island adult possum has been exposed to the parasite, but as can be seen from Fig 1.8 and from Heath *et al.*, [1999], not all have antibodies to *P. trichosuri* at observable levels. It seems reasonable to assume also that antibody levels fluctuate, as does infection, and that they may not be long lasting.

The results show some of the difficulties of working with undefined reagents, an "antigen" that is a crude protein mixture, and serum with an antibody fraction which contains many antibodies to a range of proteins with unknown cross reactivity. Sera from different infected possums appear to recognize a slightly different subset of proteins, and affinity-purified antibodies had fewer, but still multiple bands.

Detection of the antigen with the variable strength antibodies presented some problems. Bound antigens are typically visualized with chromogenic substrates [Methods in Immunology. Section 8.10]; either AEC, DAB, or 4-chloro-1-naphthol can be used when using an HRPO detection system. One nanogram of antigen can be detected normally by chromogenic methods. Antigens can also be visualized with chemiluminescent substrates,

these give enhanced sensitivity over chromogenic and even radioisotope labelling, e.g. colour development with HRPO can detect 50pg, compared with 1-10pg that may be detected using chemiluminescent methods. Difficulties in increasing the sensitivity were encountered as there were wide differences in response using different serum and insufficient quantities to use one positive serum only. Attempts were made to compensate for the differences, i.e.using both AEC and 4-chloro-1-naphthol colour substrates in Fig 2.6. It is possible that an ELISA will be developed for this reaction in the future

The labour intensive and inconclusive immuno-histochemistry was not repeated although a possible system that would have given reproducible results had been arrived at. There was some evidence that the immunogenic protein could be visualized on the intact live larva and therefore could have been present on the surface. This is one possible explanation of its exposure to the immune system of the host as the parasite moves through the tissues of the possum. The antigenic 70kD protein could not be detected in the excretory/secretory products of the cultured larvae or adults, and as it appeared unlikely that the protein of interest could therefore be isolated from the excretory/secretory products it was considered unproductive to pursue further culture work.

There were a number of proteins around 70kD on the gels and different techniques aimed at isolating individual proteins of this size were attempted without success. The results of preparative electrophoresis using the Prep Cell, however, show that samples in which one band predominates could be obtained and at least two proteins, and possibly more, of around this size reacted with affinity-purified antibodies on the immunoblots. Whether the visible band does in fact comprise one protein only cannot be known with certainty from one dimensional electrophoresis and whether it is the major protein or some minor component that reacted with the antibodies is also debatable. For instance, in lane 6, Fig 2.15 there is no staining on the gel but a reaction with antibodies can be seen in lane 6 on the immunoblots. This could indicate that some protein present at a very low concentration is immunogenic. Samples where there appeared to be a preponderance of one immunogenic protein were finally isolated and unequivocal sequence from one protein obtained. Sequence and possible identity of a second, higher molecular weight protein would have been of interest but was not obtained.

There was a high degree of homology found between the N terminal nine amino acid peptide obtained from sequencing this protein and other sequences in the databases [GenBank and Swissprot]. As this sequence was a signature for Hsp70 proteins there was a high degree of likelihood that the immunogenic protein was an Hsp70 protein from *P. trichosuri*.

Immediately, this identity helped clarify many of the experimental results. In all organisms examined so far there are more than one Hsp70 protein; in yeast there are at least ten, in *Drosophila* eight and there are at least eight known in mammals. The multiple bands seen on some Western blots can be explained by some possums making antibodies to a different subset of the Hsp70 family and it would also seem that adult worms express some different proteins from larvae.

The absence of the target molecule in the E/S products of the parasite was explained when it was identified as a heat shock 70 protein as, while they are involved in many intracellular processes they are not generally thought to be secreted [but see discussion in introduction to Chapter III]. Heat shock proteins have, however, been found in the excretory/secretory products of *Trichinella spiralis* and *T. pseudospiralis* but they were not sequenced and they were identified as heat shock proteins entirely on the basis of their upregulation after larval heat shock at 40°C and 43°C [Ko and Fan 1996]. The presence of Hsps in the excretory/secretory products of other nematodes has not been reported.

Hsp70s are known to be highly immunogenic but because of their intracellular location it is uncertain how the Hsp70 molecule is presented to the host immune system during infection. The death and dissolution of some infecting larvae on their journey through the possum is to be expected and this could expose this hidden antigen. Heat shock proteins, including Hsp70s, have been found on the surface of cells in culture which have been stressed, infected or transformed [Ponomarev *et al.*, 2000] and an extracellular sperm-binding domain on the surface of *Strongylocentrotus pupuratus* shows strong sequence similarity to Hsp70s [Foltz, 1993]. It is therefore possible that the presence of bound antibodies on the surface of the larvae does correspond to a reaction with surface Hsp70. It

is, however, possible that the antibodies, which are polyvalent, are reacting to some surface protein that is still unidentified.

Since the antigenic protein that had been identified was unlikely to be secreted, the use of its promoter in the transgenic *P. trichosuri* project had to be reconsidered. Heat shock promoters have been quite widely used in transforming other organisms and are used in constructs where gene expression is then induced by heat. This *P. trichosuri* member of the Hsp70 family, however, is most likely a cognate molecule, constitutively expressed, as it was isolated from L3 larvae prior to skin penetration so presumably in an un-shocked state. It was thought that the specific nature of the promoter would still prove to be an advantage [see Chapter I.5.2] and work to find and clone the gene continued.

Chapter III: Cloning and Analysis of a *P. trichosuri* Hsp70 Gene

Introduction: The Hsp70 Proteins

The heat shock proteins [Hsps] are abundant and ubiquitous; up to five percent of cellular protein may consist of Hsps and they are found in all organisms investigated so far, archaea, bacteria, eukaryotes and organelles, mitochondria and chloroplasts [Lindquist and Craig, 1988, Feige and Polla, 1994]. They were originally discovered as a result of greatly increased transcription of a class of proteins in cells of *Drosophila* that had undergone stress, the name-conferring heat shock for instance, but also many other kinds of physical or chemical stress. There are, however, many of these proteins that are constitutively expressed. Heat shock proteins are classified on the basis of molecular weight and amino acid homology, with new members and even new families still being assigned to this multi-function group [Easton *et al.*, 2000]. Specific members of the Hsp families in differing cell compartments carry out defined activities from a wide range of essential intracellular functions under both stressed and non-stressed conditions. The following is a brief discussion of some of these functions.

Hsps are central components of the chaperone system that protects nascent polypeptides and assists their folding, or assists re-folding of proteins that have unfolded under stress conditions [Hartl, 1996] and helps regulate the degradation of proteins through association with cofactors [Luders *et al.*, 2000]. They translocate proteins across intracellular membranes into the ER and mitochondria [Strub, 2000]. They are involved in vesicle trafficking [Ungewickell *et al.*, 1997] and signal transduction [Song *et al.*, 2001], and function in cell cycle progression and apoptosis [Beere and Green, 2001]. Also of importance is the role of Hsps in the modulation of the immune system [Multhoff *et al.*, 1998, Basu and Srivastava, 2000]. In humans they are implicated in autoimmune disease and have been associated with immune responses to cancer. In parasites, which must shift between environmental conditions and the internal environment of mammalian hosts, or conditions in different hosts, Hsps appear to play an important role in life-cycle stage differentiation and adaptation to the mammalian host; e.g. an increase in a 70 kD protein was found in *Strongyloides venezuelensis* after

a temperature shift from 25°C to 37°C, mammalian host temperature [Tsuji *et al.*, 1997]. Hsps can also act as antigens to trigger host immune responses [Polla, 1991].

Considerable work has been done on elucidating the structure and mode of action of this important group. Hsp70 proteins comprise an N terminal ATPase domain of approximately 385 amino acids followed by a substrate or peptide binding domain of about 145 amino acids then a more divergent 100 amino acid C terminal region. There may be N terminal targeting signals for those members of the Hsp70 family destined for the mitochondria or endoplasmic reticulum [ER] and a C terminal ER retention signal [KDEL] for the latter [Hartl, 1996]. All cytoplasmic Hsp70s terminate with a conserved –EEVD motif which regulates ATPase activity of the N terminal domain and the ability of the protein to interact with co-chaperones [Freeman *et al.*, 1995]. The crystal structure of both domains is known. The ATPase domain of mammalian Hsc70 consists of two globular sub domains, I and II, separated by a deep cleft in which ATP is bound [Flaherty *et al.*, 1990]. The peptide-binding domain consists of two anti-parallel β sheets of four strands each; four loops protrude from this β sandwich two of which act as the peptide binding site. There is an α helical sub-domain that appears to act as a lid [Zhu *et al.*, 1996]. Peptides are bound in an extended conformation by short, exposed hydrophobic segments of 4-5 residues that are normally buried in the core of the native protein [Rüdiger *et al.*, 1997]. This explains why Hsp70 binds to many unfolded proteins while ignoring the native protein.

The chaperone activity of Hsps involves cycles of ATP binding and hydrolysis that controls the binding and release of the substrate polypeptide. This has been described in detail in Beissinger and Buchner, 1998, and Bukau and Horwich, 1998 and others. Briefly, ATP binds with high affinity to the N terminal ATPase domain; this drives a conformational change that allows a short exposed hydrophobic region of a peptide to bind to the to the pocket of the peptide binding domain. This can occur directly or, more usually, in association with co-chaperones, the DnaJ or Hsp40 proteins, a divergent group of proteins, alike only in having a conserved J domain. Mutations in the inter-domain linker region abolish this activity [Han and Christen, 2001]. Hydrolysis of ATP is stimulated by DnaJ proteins, nucleotide exchange factors and peptide binding itself; this converts the Hsp70 to the high affinity peptide binding state and thus stabilizes the Hsp70/ADP/peptide molecule. ADP is exchanged for ATP stimulated by

nucleotide exchange factors, GrpE in *E. coli* and Bag proteins in eukaryotes, with consequent release of the peptide either folded correctly or if not, ready to be re-bound.

Stable folding to a native configuration requires at least a whole protein domain therefore all newly synthesized polypeptides are susceptible to aggregation as they leave the ribosome. Co-translational binding of chaperones is thought to provide this protection although it is subject to discussion as to what proportion of cellular proteins fold to their native conformation unassisted and what proportion are chaperoned [Jaenick, 1998]. The cell cytosol is a highly crowded macromolecular environment and this would favour aggregation over folding for a significant proportion of polypeptides without some kind of chaperone system. Some smaller [<55kD] polypeptides are folded inside the barrel-like interior of the chaperonins, GroEL or Hsp60 s or TriC family proteins; but Hsp70s are the major chaperones in the cell [Frydman, 2001].

Diverse intracellular functions are carried out by independent Hsp70 systems, possibly resulting from specific interactions between certain Hsp70s and different co-chaperones, the J proteins and other regulatory proteins such as Hip, Hop and the Bag exchange factors. These target Hsps to their specific substrates and locations [Mayer and Bukau, 1998, Takayama *et al.*, 1999].

The immunological effects of the Hsp70 proteins are surprising. These are among the most highly conserved of all proteins [Günther and Walter, 1994, Gupta and Golding, 1993] and therefore it would be expected that they should be treated as "self" and that immune reactions to Hsps would be rare. Hsp70s, however, are immunodominant and investigation of cancer antigens, the antigens of bacterial, viral and parasitic diseases, and inflammation has shown that Hsps are major targets of the cellular immune response [Multhoff *et al.*, 1998]. Hsp70s with peptides in their peptide-binding clefts are seen on the surface of some tumour cells where they give rise to a cytotoxic T lymphocyte response; Hsps with the peptides removed are non immunogenic [Srivastava *et al.*, 1998]. Therefore, Hsps may function by chaperoning antigenic peptides to the cell surface rather than acting as antigens themselves; the mechanism of transport to the cell surface is unknown although it is distinct from the MHC pathway. Antibodies to certain bacterial Hsps cross-react with Hsps from other species

implicating them in autoimmune disease; in which, confusingly, they appear to be both antigenic and protective [Multhoff *et al.*, 1998, van Eden *et al.*, 1998].

Finally, Hsps are important signals in the "danger" theory of the immune response [Matzinger, 1994]. This postulates that the way a cell dies determines whether or not an immune response is induced. Necrotic death resulting from bacterial or viral infection or inflammation results in high level of Hsps which constitutes a strong danger signal; the Hsp/peptide complex may give rise, either directly or indirectly, to the co-stimulatory signal on the antigen presenting cell. Apoptotic cell death, "quiet" death, as occurs in cell turnover, tissue remodelling etc. is not accompanied by raised Hsps or an immune response [Todryk *et al.*, 2000].

Commonly, it has been considered that the diverse function performed by the Hsp70s, and heat shock proteins in general, are all intracellular. There is evidence, however, that heat shock proteins may also act as intercellular signalling proteins by stimulating cytokine production and by acting directly on cell surface receptors [reviewed in Ranford *et al.*, 2000]. The evidence is more abundant in the case of Hsp60 – chaperonin- but some evidence exists for Hsp70 also. Exogenous Hsp70 bound to the plasma membrane of cultured human monocytes activated inflammatory cytokine production by stimulating two cell-signalling pathways [Asea *et al.*, 2000] and there is the presence of Hsp70 found on the surface of stressed, infected or transformed cells already mentioned [Ponomarev *et al.*, 2000]. Bacteria secrete quite large amounts of Hsps and some eukaryotic cells have been shown to secrete Hsp60 and Hsp10 [Ranford *et al.*, 2001]. There is as yet no known mechanism to explain the secretion of molecular chaperones.

As discussed, the promoter for a *P. trichosuri Hsp70* gene would seem to be a good candidate for transgene expression in *P. trichosuri* and the gene itself would be of interest, therefore delineating these sequences was given priority.

Methods:

3.1] Preparation of genomic DNA from *P. trichosuri* L3 infective larvae.

Genomic DNA was prepared from L3 infective larvae obtained as described in Chapter II.1.4. Approximately 1 ml of L3 infective larvae, snap frozen without protease inhibitors, were thawed, centrifuged at 2000 rpm for 20 sec and as much water removed as possible. The pelleted worms were digested with 2 ml of worm lysis buffer for 1hr at 65°C with occasional gentle mixing. [Worm Lysis Buffer: 100mM Tris-HCl pH 7.5, 200mM NaCl, 50mM EDTA, 1%SDS, 1% 2-mercaptoethanol, Proteinase K at a final concentration of 100ug/ml]. A further aliquot of Proteinase K was added and incubation was continued for a further hour until all visible worm debris had been digested. An equal volume of phenol [Gibco buffer saturated phenol pH 7.4-7.9] was added to the digest and slowly inverted for 15 min then centrifuged at 2000g for 15 min. The aqueous phase was removed, a further ml of worm lysis buffer added to the phenol phase and digestion and centrifugation repeated. The combined aqueous phase was extracted with an equal volume of phenol/chloroform/isoamyl alcohol [25:24:1], then chloroform/isoamyl alcohol. The DNA was precipitated with cold absolute ethanol, washed with 70% ethanol and re-suspended in TE buffer.

3.2] Isolation of clones containing the gene of interest from *P. trichosuri* genomic library.

3.2.1 Making the *Hsp70* Probe

Primers, designated PtHsp70-A and PtHsp70-B, were designed based on the *C. elegans* Hsp70A gene [Snutch T P *et al.*, 1988 GenBank Accession number M18540]. These were based on the more highly conserved N terminal part of the gene, with degeneracy at position 3 from the 3' end of both sense and antisense primers; the sequences are shown in Table 3.1. PCR reactions were carried out using Expand™ High Fidelity PCR System [Boehringer Mannheim], using the genomic DNA prepared in IV.1 as template. Primers were used at a final concentration of 3 μM and Mg^{2+} ions at a concentration of 3.5mM. A positive control using highly conserved generic nematode primers was

Table 3.1

Pt Hsp70 primer sequences

Primer names	Primer sequence	Used for
Pt -A	5' gtt cag tct gat atg aag cay tgg ccn t	degenerate sequence based on C. elegans A
Pt -B	5' gat tta act tcg aag att ccr tcn tcd at	labelled probe for library screening/ sequencing
Pt -Nt	5' ctt gcg gcc gct gga aca gtg aca aca gca tc	PCR of fragment of clone DNA, sequencing
LMB-T3	5' aat taa ccc tca cta aag gga acg a	long PCR of clone inserts, template for sequencing
LMB-T7	5' cgt aat acg act cac tat agg gcg a	reverse primer, long PCR of clone insert
Pt -C	5' caa gac att ctt ccc aga ag	sequencing, sense strand, genomic and cDNA,
Pt -D	5' aga ctg gat att tct cga tat g	sequencing, antisense strand
Pt -E	5' gat gct aag atg gac aaa gg	sequencing, sense strand
Pt -F	5' gct tga cag cac caa tct gct	sequencing, sense strand, and cDNA
Pt -G	5' gtt gaa atg cct cca tta cct c	sequencing, antisense strand
Pt -H	5' gga tag agt cca taa aaa ggc	sequencing, antisense strand
Pt -J	5' gcc ttt tta tgg act cta tcc	sequencing, sense strand
Pt -K	5' cgg tat tga tct cgg tac cac	sequencing, sense strand
Pt -L	5' gaa aga cgt cct ttg tcg ttg g	sequencing, antisense strand, and cDNA
Pt -M	5' gaa ctt ttg gaa tac ggg ttg atc c	sequencing, antisense strand
Pt -N	5' gaa aat cct ttc aac tgc aat cg	sequencing, antisense strand
Pt -P	5' cag tca aaa tgg aga tct act g	sequencing, antisense strand
Pt -Q	5' gag gta gga aaa atg agg tgt g	sequencing, sense strand
Pt -R	5' ggg att agg gag tgt aac act tc	sequencing, sense strand
Pt -S	5' cca ggt att cct ggt tca cca g	sequencing, sense strand
Pt -T	5' gca act tct ttt ctt act tca ctc	sequencing, sense strand
Pt -U	5' gaa ggt cgt agc aca ttc gtc aac	sequencing, sense strand
Pt -prm1	5' ctt gcg gcc gcg ttc acc aga ttt tcc ttg	PCR promoter fragment
Pt -prm2	5' gat cgg cgc gcc ttt ttc tta tta aaa tat tag c	PCR promoter fragment
Pt -ex1	5' gat cgg cgc gcc ata tgg tta aag cta atg cta tc	PCR coding sequence, sequencing, cDNA
Pt -ex2	5' ctt gcg gcc gcg tca act tct tca att gtt g	PCR coding sequence, sequencing cDNA

included. All PCR reactions were carried out using a MJ DNA Engine; the profile for this reaction was: denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 30 sec, for 30 cycles. The PCR product was purified using High Pure PCR Purification kit [Boehringer Mannheim].

The purified product was radioactively labelled by random oligonucleotide-primed synthesis using the RTS RadPrime DNA Labelling System [Life Technologies]. 26ng of purified PCR product in TE buffer was denatured and added to the RadPrime reaction mixture containing 60µg/ml random octamer primers, 10µM dATP, dGTP and dTTP, 3-6 units/µl Klenow fragment. 5µlof [α- ^{32}P]-dCTP having an activity of 10uCi/µl was added [NEN Easytide ^{32}P Nucleotides, Life Technologies], the reaction was incubated at 37°C for 10min and stopped with 2µl 0.5M EDTA. The reaction mixture was again denatured by heating at 95°C for 5min and added to the library filters.

3.2.2 Screening a *P. trichosuri* genomic library

An existing *P. trichosuri* library, which had been made commercially from *P. trichosuri* infective L3 genomic DNA, was screened [Gruenberg *et al.*, 1999]. Inserts of 9-23 Kb were cloned into the *BamH I* site of the replacement cloning vector, Lambda DASH II® [Stratagene]. The library was titrated and the recombinant phage plated out on host strain XL1-Blue MRA(P2). Pfu/ml was not estimated but 1µl of a 10^{-2} dilution of the library was used, giving about 1500 discrete colonies per plate; 10 10x10cm agar plates were used. Approximately $1.5x10^4$ clones were screened.

Plaque DNA was transferred to sterile nitrocellulose filters [Hybond N+, Amersham]; the filters were left in contact with the agar for 2min, duplicate filters for each plate were left on for 4min. After transfer, the filters were denatured with 0.5M NaOH, 1.5M NaCl, neutralized with 0.5M Tris pH7.0, 1.5M NaCl, 1.0M EDTA, washed with x20 SSC [see Appendix 3], DNA was then UV linked to the filter in the Stratalinker 1800 at a setting of 120,000 micro joules.

Hybridization of plaque DNA to the radioactively labelled probe, made in 4.2.1, was carried out as follows: filters were equilibrated with a hybridization solution [see Appendix 3] at 42°C for 1hr. The labelled probe was added and left to hybridize at

42°C overnight. The filters were washed in 2X SSC buffer for three low stringency washes and in 0.2X SSC for two high stringency washes at 42°C. They were then autoradiographed [Kodak X-OMAT-AR film] for 24hr and developed. Clones that were positive on both the primary and duplicate film were identified and ten of these were purified by two further rounds of screening. Four of the most strongly hybridising clones were used for subsequent procedures.

3.3] Preparation and characterization of DNA from selected phage

Phage, from clones, 5.1, 5.2, 7.1 and 7.3 which had been identified as containing all or part of the *P. trichosuri Hsp70* gene, were amplified in the host cell XL1-Blue MRA(P2) and XL1-Blue MRA without the P2 lysogen [as this may give a higher titre of phage]. Lambda DNA was extracted by one of two methods: precipitation with zinc chloride followed by Proteinase K digestion and phenol/chloroform/isoamyl alcohol extraction [Su *et al.*, 1998]; or PEG precipitation and anion exchange chromatography using a QIAGEN® Lambda midi kit.

DNA from all four clones was digested with 50µl of *Not1* in Buffer3 [New England Biolabs]. Further aliquots of each DNA were digested with *BamH 1*, *EcoR 1*, *Hind III* and *Xho I* [NEB] according to the manufacturer's instructions. The digestion products were run on a 0.6% agarose gel at low voltage [35v] for two hours, transferred by Southern blotting to nitrocellulose [Hybond N^+, Amersham] according to standard protocols [Current Protocols in Molecular Biology, 9.34]. The DNA fragment, Pt-A/Pt-B was radioactively labelled as described above [2.1] and allowed to hybridize to the digestion products. The membrane was autoradiographed for 1hr and the size of positively labelled fragments determined by reference to the original agarose gel. A restriction map of each clone was attempted.

3.4] Long Template PCR of Lambda clone DNA

Primers designated LMB-T3 and LMB-T7 were designed from T3 and T7 promoter sequences plus some sequence of the Lambda DASH II vector distal to the clone insert. The increased primer length was necessary to increase annealing temperature to [theoretically] 70°C in order to achieve accurate priming and eliminate non-specific binding, with consequent amplification of shorter non-specific products that can be of concern in amplifying fragment of more than 2-3kb in length. The sequences are shown in Table 3.1. DNA was prepared from clone 5.1 and 7.1 using the QIAGEN® midi kit; this was of high purity with little or no RNA present and approximately 10-20 ng was used as a template in the Long PCR reaction.

PCR reactions were carried out using the Expand™ Long Template PCR System [Roche] according to the manufacturer's protocol for a target product of 5-20Kb; using the dNTP nucleotide mix at a final concentration of 500µM, primers at 0.2µM, PCR buffer1 with a Mg^{2+} concentration of 1.75mM, template DNA at 10-20ng and Expand Long Template enzyme at 2.5units. The temperature profile of the PCR reaction was as shown in Table 3.2.

In order to determine the orientation of the DNA fragment Pt-A to Pt-B in the insert T3-T7, PCR reactions using the primer pairs LMB-T3 /Pt-A, Pt-B and LMB-T7 /Pt-A, Pt-B were carried out. Also, a *P. trichosuri* specific primer, based on the Pt-A to Pt-B sequence, Pt-Nt was made. Pt-Nt had a high annealing temperature [theoretically 70°C] and a 5' *NotI* restriction site and was used to obtain smaller fragments of the T3-T7 insert, which were originally intended for sub cloning. These reactions used the same PCR reagent concentrations and temperature conditions as above.

The reaction products from one clone, 5.1, were purified; suspected double band products were re-run on gels, gel cuts taken, the products extracted from the gel [Concert Gel Extraction System-Life Technologies] then re-amplified under the original PCR conditions except 25 cycles only of amplification were performed. The products of this second round of PCR were purified using High Pure PCR Product Purification Kit [Roche] and used as template for sequencing reactions at a concentration of 20ng/ul.

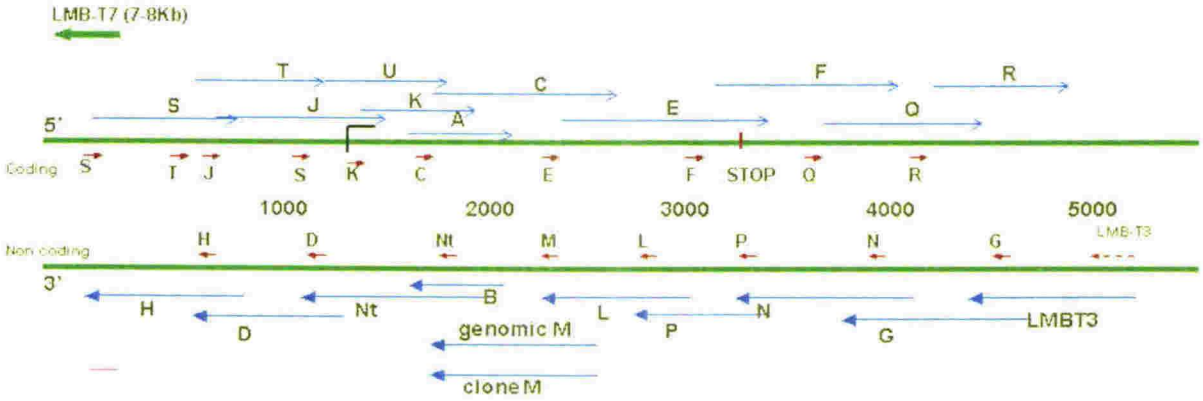
Table 3.2 Temperature profile of the long PCR reaction used for amplifying the insert of clone 5.1

Step	Temperature °C	Time	No. Cycles
1. Initial denaturation	93	2 min	
2. Denaturation	93	10 sec	
3. Annealing	65	30 sec	X10
4. Elongation	68	6 min	
5. Denaturation	93	10 sec	
6. Annealing	65	30 sec	X19
7. Elongation	68	6 min + 10 sec added per cycle	
8. Final extension	68	7 min	

3.5] DNA Sequencing

MUSeq DNA Analysis Service at Massey University [Palmerston North, New Zealand] carried out sequencing reactions using a Dye Terminator Cycle Sequencing Ready Reaction Kit then an ABI Prism 377 DNA Sequencer [Perkins Elmer Pty Ltd]. Forward and reverse strand sequencing was carried out by primer walking; a series of primers was designed from a preceding established DNA sequence, and contiguous and unambiguous sequence was obtained from the T3 promoter at one end of the inserted *P. trichosuri* DNA for a distance of 5kb. One primer pair was used to amplify a segment of genomic DNA and the sequence of this was compared with that of the same segment from the clone DNA. Primer sequences are shown in Table 3.1 and the sequencing strategy is shown in Fig 3.1.

The sequences were analysed by the Wisconsin Package version 9.1 software, Genetics Computer Group, Madison, Wisconsin. The sequence data files were entered into the Fragment Assembly System, overlapping sequences were aligned and merged into a series of contiguous sequences [contigs]. The sequences were edited in the contigs, the degree of overlap being such that all base positions could be unambiguously assigned. Both forward and reverse strands were sequenced and compared.



5.04kb insert DNA sequenced by primer walking

Fig 3.1 Sequencing strategy for the insert of clone 5.1
Location of PCR primers used in sequencing 5kb of insert DNA from clone 5.1. Arrows indicate the direction in which DNA amplification was initiated from each primer. The START and STOP positions of the open reading frame are indicated.

Results:

One of the immunogenic 70 kD proteins of *P. trichosuri* had been identified as a heat shock 70 protein. The gene for this protein and surrounding sequences were obtained by screening an existing genomic library with a radioactively labelled oligonucleotide probe. As the genes for the Hsp70 proteins are highly conserved, primers based on a *C. elegans* orthologue could be used to amplify a probe fragment from *P. trichosuri* DNA.

3.2]. Isolating clones containing the gene of interest from *P. trichosuri* genomic library.

3.2.1 Making the *Hsp70* probe

Genomic DNA was extracted from *P. trichosuri* L3 infective larvae and used to make a probe from the *Hsp70* gene.

The positive control, a 500bp portion of the internally transcribed spacer [ITS] of nuclear ribosomal DNA, was amplified at a magnesium concentration of 1.5mM. A higher Mg concentration of 3.5mM was optimal to obtain a product using the Pt *Hsp70* primers. At this concentration a 418bp piece of DNA was amplified, which was the size expected by comparison with the *C elegans Hsp70* gene. This DNA fragment was sequenced and the nucleotide sequence showed 74% homology with the C. elegans *Hsp70*A gene [Snutch *et al.*, 1988]. 26 ng of this DNA was radioactively labelled and used to screen the *P. trichosuri* library as described below; the specific activity of the probe was not determined but there was a discernible level of radioactivity on the washed library filters after hybridisation.

3.2.2 Screening a *P. trichosuri* genomic library

Recombinant phage were grown overnight to give large but not confluent plaques. This gave a plaque density of about 1500/plate and a total of about 1.5×10^4 screened. This is less than theoretically desirable [2.8×10^4]; in general to have a 99% chance of isolating a desired sequence, the number of clones screened should be such that the total number of base pairs screened [clones x average size of insert] should represent a 4.6 fold excess over the number of base pairs in the total genome of the organism: Therefore for

P=99% N = G/I x4.6 where N=number of clones, G=number of base pairs in the genome, I=average number of base pairs in the inserts of the library. (Seed *et al.*, 1982). Despite the low number screened, at least 17 clones were found to be positive on both filters on primary screening.

Ten of the positive clones were purified by two further rounds of screening. By the tertiary purification round, two clones had been lost and almost all the plaques were positive on the autoradiograph. Three plaques from each of four strongly hybridising clones were excised and kept as phage stock. These were labelled for the primary plate from which they were taken: clones 5.1, 5.2, 7.1, 7.3 were kept and used subsequently.

3.3] Preparation and characterization of DNA from selected phage

Initial titration of the phage stocks showed lower than expected titres, approximately 10^5pfu/ml, and subsequent titrations were variable though still far lower than the 10^{10} pfu/ml recommended for efficient DNA extraction. Therefore large volumes of phage were used in order to reach the recommended pfu level and lyse most cells during DNA amplification. In two of the preparations the final amount of phage was measured and 10^{10}-10^{11} pfu were added to 50ml cultures with cell grown to an OD of 0.7-0.8. These phage levels should have given yields of 150-300ug [Su *et al.*, 1998] but repeated preparations, ranging in volume from single plate to large 500ml flask, yielded no more than a few nanograms of lambda DNA. Particularly disappointing were the results using the QIAGEN® Lambda midi kit where analysis of fractions taken throughout the preparation showed that there was sufficient propagation of lambda but that either the phage did not precipitate with the poly ethylene glycol and/or there was incomplete phage lysis. Treatment with Proteinase K to ensure complete digestion of the phage head did not improve yield and the manufacturers were unable to suggest reasons for or ways to improve the precipitation step.

Initially, small amounts of DNA were obtained that were used for restriction digestion and Southern blotting. Partial digestion resulted and the labelled probe hybridized strongly with several fragments on Southern blotting; however, the hybridization pattern obtained did not allow a coherent restriction map of the insert to be derived [results not shown]. The continuing very low yields of λ DNA meant that repeat restriction

mapping of the clone would be unprofitable, as insufficient DNA remained to sub-clone into a sequencing vector. The initial intention, therefore, of identifying then cloning a smaller fragment into a vector suitable for sequencing was abandoned. Instead it was decided that credible gene sequence could be realized using a PCR strategy.

3.4] Long Template PCR of Lambda clone DNA.

In the library, the *P. trichosuri* DNA fragments are cloned into the *BamH I* sites of the Lambda DASH II vector; these are flanked by T3 and T7 promoters and more distally by *Not I* sites. Initial attempts at amplifying the entire insert were made using existing T3 and T7 promoter primers as suggested by the library manufactures; no product, however, was obtained, probably due to the annealing temperatures of these primers, 52°C and 56°C respectively, being too low. Therefore primers LMB-T3 and LMB-T7, which included part of the *Not I* site and had theoretical annealing temperatures of 70°C, were used. Also, Pt-Nt, a *P. trichosuri* specific primer, based on the Pt-A to Pt-B sequence, with a high annealing temperature and a 5' *NotI* restriction site. The conditions for the PCR reaction were optimized and, using DNA from clone 5.1 as template, a product of 12-13kb corresponding to the entire insert of the clone was consistently obtained.

Primer pair LMB-T7/Pt-Nt gave rise to a product of approximately 8kb and LMB-T3/Pt-A gave a product of 3.3kb in size shown in Fig 3.2; this gives the orientation of the probe fragment Pt-A to Pt-B as shown in Fig 3.3. Other, lower intensity bands were seen less consistently on gels, particularly from reactions performed under conditions of lower stringency; i.e. Mg^{2+} of 3.5mM and 29 rather than 25 cycles of amplification, these were thought to be products of non specific priming due to the large size of the template such as are known to occur in long PCR reactions [Cheng *et al.*, 1994]. There was, however, a reasonably consistent product of the reaction using LMB-T3 and Pt-Nt seen at about 6kb; this is inconsistent with the orientation shown above and indicates a possible second Nt-like priming site in a part of the DNA upstream of the region that was finally sequenced. Subsequent sequencing confirmed the orientation was as shown. Further investigation of this product was not carried out as sequences that were obtained gave perfect contiguous overlapping sequence in accord with the initial orientation.

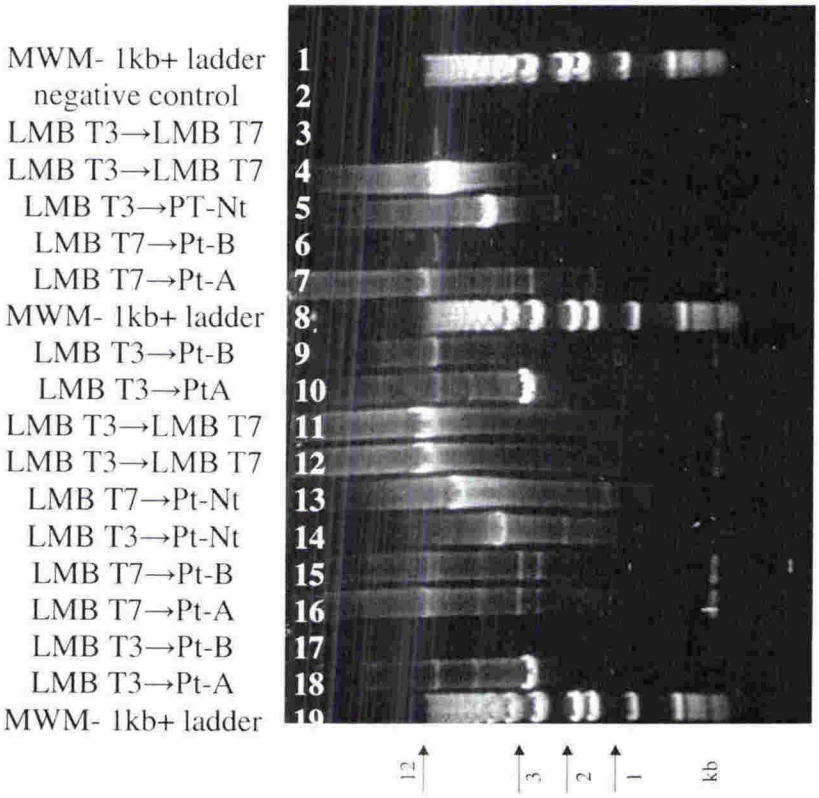


Fig 3.2 Products of long PCR reactions of clone 5.1 from the *P. trichosuri* genomic library.

The entire insert of the clone was amplified using the flanking primers LMB-T7 and LMB-T3. The LMB T7→LMB T3 fragment consistently amplified at 12- 13kb [lanes 3, 4, 11 and 12].

Attempts to define the orientation of the insert were made by reactions with either of the flanking primers and one of the internal primers Pt-A, Pt-B or Pt-Nt. The fragment LMB T7→Pt-Nt consistently amplified at 7-8kb, [lane 13], LMB T3→Pt-A gave a product of 3.3kb, [lane 10 and 18], LMB-T7→A [lane 7, 16] and LMB-T3→B [lane 9, 17] gave no clear product which suggests the orientation shown in Fig 3.3. But LMB T3→Pt-Nt also gave a product of approximately 6kb, [lanes 5, 14], which is inconsistent with this orientation and the reaction LMB-T7→B [lane 6, 15] gave no product of any size.

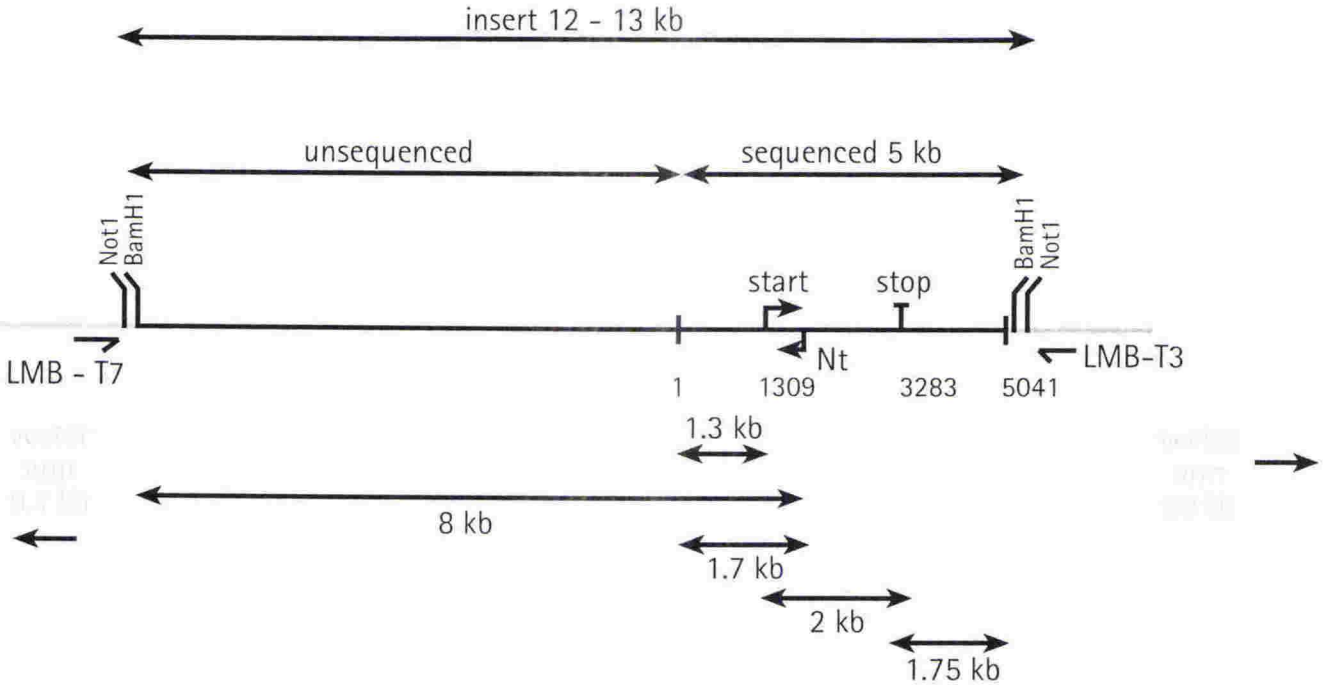


Fig 3.3 Diagram of the insert of clone 5.1 from the genomic library of *P. trichosuri.*

Clone 5.1 consisted of 12-13 kb of *P. trichosuri* genomic DNA cloned into the BamHI site of the LAMBDA DASH II vector. 5kb of insert DNA was sequenced from nucleotide 1 to 5041 at the 3' end of the insert. The open reading frame of the *Hsp70* gene from *P. trichosuri* is shown from nucleotide 1309 to 3283. The positions of primers LMB-T7, LMB-T3 and Pt-Nt, that were used to amplify template DNA for sequencing, are shown.

Products from several of the LMB-T7, Pt-Nt reactions showed double bands [Fig 3.4]. These were purified, amplified again and both the 7.5 and 8kb products were sequenced from the Pt-Nt primer to ensure that there was only a single product [Fig 3.5]. The samples had identical sequence for 600bp, from bp1773 to 1096 [antisense strand] .This sequence reads through the single intron; different genes would almost certainly show some differences in this region. Therefore it was accepted that these were products from the same gene not from two homologous *Hsp70* genes in tandem. The difference in size on the gel might have been due to some secondary structure in the fairly long product which might have influenced the folding, and therefore the speed of migration of some molecules but not others.

3.5] DNA Sequencing

The purified fragment LMB-T7 to Pt-Nt of 8kb in size was use as a template for sequencing from primers: Pt-D, H, J, K, Nt. The LMB-T3, Pt-A fragment was used as a template for sequencing from primers: Pt-C, E, F, G, L, N. The entire insert LMB-T3, LMB-T7 was used for primers: Pt-N, P, Q, R, S, T, U. As a check on the accuracy of the sequence derived from the PCR product, the primer pair Pt-J, Pt-L was used to amplify similar regions from both genomic DNA and lambda clone 5.1 DNA. A similarly sized product of about 2kb was seen from both templates and was sequenced from primer Pt-M; there was 100% identity for 600bp. At this region of the gene there were five sequences with some degree of overlap, which gave identical results. This indicates that the PCR product is an accurate template and we can have a high level of confidence in the sequence that has been produced.

The sequence fragments were aligned into a contiguous piece, 5kb, long of unambiguous sequence; this is shown in Fig 3.6. There was an ATG start codon at position 1309 followed by an open reading frame of 1974 base pairs that closed with the stop codon TAA at position 3283; the open reading frame was interrupted by a single intron from position 1409 to 1451. The translational start is preceded by four adenine residues, which is consensual in *C. elegans*. The TAA translation stop codon is used in 61% of *C. elegans* genes and is preceded by a/t, t/c and followed by c, t; [Blumenthal

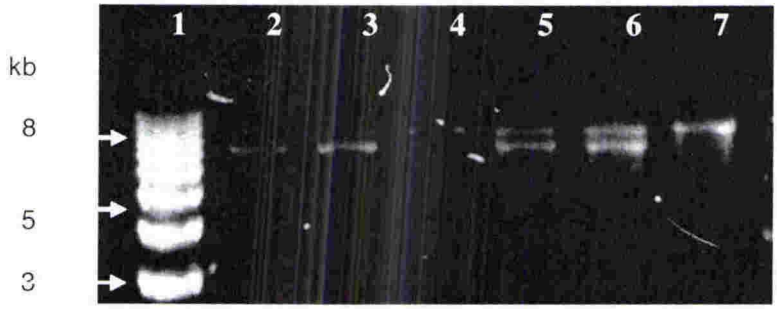


Fig 3.4 PCR reaction products of the amplification of the LMB T7→Pt-Nt fragment.

Clone 5.1 DNA was amplified using primer pair LMB T7/Pt-Nt. Two reaction products were obtained [lanes 2-6] of approximately 7.5-8 and 8.5-9kb in size. Lane 7 has only a single product [some are faint in this reproduction of the photograph].

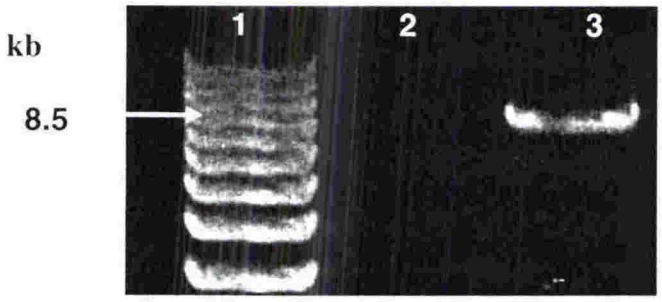


Fig 3.5 Purity of sequencing template DNA, LMB T7→Pt-Nt.

One of the two PCR products shown in Fig 3.4 was excised and extracted from the gel, reamplified under similar conditions and re-purified using High Pure PCR Purification Kit [Roche]. The result shows a single product only [lane 3]. This DNA is used as template DNA for sequencing reactions. Lane 1 is the 1kbplus molecular weight ladder, and lane 2 is the negative control.

and Steward, 1997] the Pt*Hsp70* sequence is A, C, TAA, T, T, showing good agreement with the consensus.

Translation of the sequences 1309---1408 and 1452---3289 gave a protein of 644 amino acids with a calculated molecular mass of 70.15kD and a theoretical pI of 5.23. There was a 74% likelihood of this being a cytoplasmic protein [kNN prediction] reinforced by the presence of an EEVD motif, a highly conserved sequence in cytoplasmic Hsps. Three signature patterns have been derived for the Hsp family of proteins: [Prosite PS00297, PS00329, PS01036], the primary sequence of this protein showed, as expected, all three. Comparison of the amino acid sequence for Pt Hsp70 protein with that from *C. elegans* showed an 85.9% similarity [using ALIGN GeneStream]. Comparison of the nucleotide sequence for the coding regions of *P. trichosuri Hsp70* and *C. elegans Hsp70*-A gave a similarity of 74.9% [using Gap, GCG].

Analysis of the 5' upstream region of the gene showed a sequence, designated HSE1, from position 974-987 [-335 to -322 from the first position of the start codon] which showed 100% homology to the heat shock regulatory element [HSE], the hyphenated dyad 5'-C--GAA--TTC--G-3' [Pelham, 1982, 1985]. Other HSE-like elements were found: HSE2 at 1122-1135 having 88% homology and two overlapping elements, from 1051-1064 and 1061-1074 having 88% and 75% homology respectively. The region upstream from the ATG start codon was searched for promoter elements having the sequence TATAA, the *C. elegans* consensus TATA box sequence. These were found at nucleotide position 135, 180, 677, 854, 1001, 1023, 1195, 1209, 1231; this last being 78 nucleotides upstream from the start at 1309. It is possible that a variation on the *C. elegans* sequence could act as the TATA box in *P. trichosuri*; TATAT was found at position 1260, 49 base pairs upstream and AATAT found at 1290, 19 base pairs upstream from the start codon. No CAAT box with unequivocal homology to the sequence GGCCAATCT was found, but CAAT patterns were found at nucleotide position 36, 558, 733 and 805.

In many nematodes messenger RNA molecules are transpliced with one of two conserved 22 nucleotide sequences termed spliced leader sequences, SL1 and SL2 [Nilsen, 1993]. In *C elegans* about 70% of gene products are transpliced [Zorio *et al.*, 1994] and in *Ascaris lumbricoides* about 80-90% [Maroney *et al.*, 1995, abs.]; it would

therefore seem likely that it is a feature of nematode RNA processing in general and the 5' upstream region of this gene was searched for evidence of transplicing. The 3' spliced leader acceptor site is generally within 10 base pairs of the initial methionine codon and has a consensus sequence which is the same as the 3' consensus for *cis* spicing of introns. It is possible that this gene is transpliced to the site 6 nucleotides upstream from the start codon although the sequence does not show high homology, or to the –60 site that has good homology but is rather too far upstream. Investigation of the mRNA for this gene would be necessary to ascertain if this is so.

No heat-shock protein signatures or HSE sequences were found in the 3' downstream sequence following the stop codon.

The sequence for the entire 5kb piece of DNA comprising a 5' upstream region containing putative promoter elements, an ORF of 2kb and the 3' downstream region were submitted to GenBank, Accession no. AF278536. This was the first sequence for this organism in the database.

The sequence was compared with *Hsp70* sequences of other nematodes obtained from the GenBank database. The amino acid sequence alignment among these sequences is shown in Appendix 4 [using the multiple comparison programme GCG PILEUP], this shows an extremely high degree of homology across the entire protein. The degree of similarity and identity between the Hsp70 amino acid sequence of *P. trichosuri* and those of other nematodes is also extremely high; identity from 82-90% and similarity from 90-95%; the values are shown in Table 3.3. These values are so uniformly high that it is not possible to infer a closest relative from them.

***Parastrongyloides trichosuri* – PtHsp70**

```
                .         .         .         .         .         .
   1  TGGTTCACCAGATTTTCCTTGATTTCCTTGAATCCAATTTCACCTTCTAAGCCTTGTTCT   60
                .         .         .         .         .         .
  61  CCCATATCTCCTGATTTCCCGGGTAAACCTTGTTTACCTTTAACACCTGTTTCACCTCTA  120
                .         .         .         .         .         .
 121  GATCCCCTTTTACCTATAAATAGTATATTTAAATTGTTTCATAATAAGTTACATACCTTT  180
                .         .         .         .         .         .
 181  ATAACCTGCATAACCAGGCCGTCCTGCTGGACAACGTTCACATGTTCCATAAATATCATT  240
                .         .         .         .         .         .
 241  TTCGTCAAAATCATTACCTTTTGTGCCATCTTTCCCGGGCTCACCATTTAAACCCACCTC  300
                .         .         .         .         .         .
 301  TCCTGCTAAACCGGGAAGACCTTTTGGTCCAGGTAGACACCTACTTTTTATATTTAGTTT  360
                .         .         .         .         .         .
 361  TTTATTACCAAGATGTTCACTCTTTCTATTAATTACATACTGACCTTCCCTAACAAATCT  420
                .         .         .         .         .         .
 421  TATGTTTTTATTCACATTAAGATCAGCAACTTCTTTTCTTACTTCACTCCACATTTCATT  480
                .         .         .         .         .         .
 481  GCTAATTTCCATATACCTTTTTCTCTCCGCATTTATTTCATCTTCCATCACATAAAGTTG  540
                .         .         .         .         .         .
 541  GCAAAATAATATTGGCACAATAATTAAAAAACAACAAAATGCAAATGTAGAAAGAGATAA  600
                .         .         .         .         .         .
 601  GCCAATATGAATAGCCTTTTTATGGACTCTATCCATTATTATCAAATGACAAATTTTATG  660
                .         .         .         .         .         .
 661  ACATATTTCATATTTATATAAAAAAAACGAGGATTTAGAACATGTTTCTTTGGTTGATCA  720
                .         .         .         .         .         .
 721  TTATCATATCTCAATTCATATTATCTTCTGATAACACCTACAGATAGTATTATTGTTAAT  780
                .         .         .         .         .         .
 781  TTTCTTAATTTTAAATCTTGTCATCAATATTTCCAAAATGTGCATTTCTTTGTGAAAAAA  840
                .         .         .         .         .         .
 841  AACAACTACATTTTATAAATGATTGAACATACGCATTCCCTAACATACACATATGTATAT  900
                .         .         .         .         .         .
 901  ATGTGCGTCCTAACATATTTTTTCTACTTTGCTGTATGTACATATTTAAAAAAGCGCCAC  960
                .         .         .         .         .         .
 961  ATACTTTACCCTTCTAGAACATTCTAGACTGTACTATATATATAATCCCAACGCGAACAT 1020
                .         .         .         .         .         .
1021  TATATAATGCAAGCTGACTGGCCGCCTCTTTTAGAATGTTCCAGAAGGTCGTAGCACATT 1080
                .         .         .         .         .         .
1081  CGTCAACCGCCTTTTATATTGTAGACCTGATGAAATTCGTTCTAGAAGCTTCTACATAGT 1140
                .         .         .         .         .         .
1141  ACACTTCTGACGAGTCATATCGAGAAATATCCAGTCTTTGCATTGCTTTTCATATATAAC 1200
                .         .         .         .         .         .
1201  ACCGCTTTTATAATTTTCATATCATCACACTATAATTTATTATTTCAGAATAACTTTGTT 1260
                .         .         .         .         .         .
1261  ATATTCCACAACAGGTTTAAATTTTTGCTAATATTTTAATAAGAAAAAATGGTTAAAGCT 1320
   1                                                   M  V  K  A     4
                .         .         .         .         .         .
1321  AATGCTATCGGTATTGATCTCGGTACCACATATTCATGTGTAGGTGTTTTCCAACATGGA 1380
   5  N  A  I  G  I  D  L  G  T  T  Y  S  C  V  G  V  F  Q  H  G    24
                .         .         .         .         .         .
1381  AAAGTTGAAATTATTGCTAATGACCAAGGTATGTTTTAGAATTATTATTTTAAAATAATC 1440
  25  K  V  E  I  I  A  N  D  Q                                    33
                .         .         .         .         .         .
1441  ATTATTTTTAGGTAATCGTACAACACCATCATACGTTGCCTTCACTGACAGTGAAAGACT 1500
  34           G  N  R  T  T  P  S  Y  V  A  F  T  D  S  E  R  L    50
                .         .         .         .         .         .
1501  TATTGGAGATGCTGCCAAAAATCAAGTTGCTATGAACCCACAAAACTCTGTCTTTGATGC 1560
  51   I  G  D  A  A  K  N  Q  V  A  M  N  P  Q  N  S  V  F  D  A    70
```

```
1561  TAAACGTCTTATTGGAAGAAAATTCGACGAAGCTGCTGTTCAAGCTGACATGAAACATTG  1620
  71    K  R  L  I  G  R  K  F  D  E  A  A  V  Q  A  D  M  K  H  W    90

1621  GCCATTCAAAGTTATATCTGCTGATGGAGGACGTCCAAAGGTCCAAGTTGAATTCAAAGG  1680
  91    P  F  K  V  I  S  A  D  G  G  R  P  K  V  Q  V  E  F  K  G   110

1681  AGAAGTCAAGACATTCTTCCCAGAAGAAATTTCATCTATGATCCTTACTAAAATGAGAGA  1740
 111    E  V  K  T  F  F  P  E  E  I  S  S  M  I  L  T  K  M  R  E   130

1741  AACTGCTGAAGCTTATCTTGGACAAACTGTTACTGATGCTGTTGTCACTGTTCCAGCTTA  1800
 131    T  A  E  A  Y  L  G  Q  T  V  T  D  A  V  V  T  V  P  A  Y   150

1801  TTTCAACGACTCTCAAAGACAAGCTACAAAAGATGCTGGAGCCATTGCTGGTCTTAATGT  1860
 151    F  N  D  S  Q  R  Q  A  T  K  D  A  G  A  I  A  G  L  N  V   170

1861  TTTACGTATTATTAATGAACCAACAGCCGCTGCTATTGCTTATGGTTTAGATAAGAAAGG  1920
 171    L  R  I  I  N  E  P  T  A  A  A  I  A  Y  G  L  D  K  K  G   190

1921  AGTTTCTGAACGTAATGTTCTTATTTTTGATCTTGGTGGTGGTACTTTTGATGTATCAAT  1980
 191    V  S  E  R  N  V  L  I  F  D  L  G  G  G  T  F  D  V  S  I   210

1981  TCTTACTATTGAAGATGGAATTTTTGAAGTTAAGTCAACTGCTGGAGATACTCATTTAGG  2040
 211    L  T  I  E  D  G  I  F  E  V  K  S  T  A  G  D  T  H  L  G   230

2041  AGGTGAAGATTTTGATAACCGTATGGTTACTCACTTTGTTAATGAGTTTAAAAGAAAGCA  2100
 231    G  E  D  F  D  N  R  M  V  T  H  F  V  N  E  F  K  R  K  H   250

2101  TAAGAAAGATTTATCAGCCAATCCACGTGCTCTCCGTCGTTTAAGAACTGCTTGTGAACG  2160
 251    K  K  D  L  S  A  N  P  R  A  L  R  R  L  R  T  A  C  E  R   270

2161  TGCTAAGAGAACACTTTCTTCATCAACTCAAGCATCAATTGAAATTGATTCACTTTTTGA  2220
 271    A  K  R  T  L  S  S  S  T  Q  A  S  I  E  I  D  S  L  F  D   290

2221  TGGAATTGATTTCTACACCAACATTACTCGTGCTCGTTTTGAAGAACTTTGTGCTGATCT  2280
 291    G  I  D  F  Y  T  N  I  T  R  A  R  F  E  E  L  C  A  D  L   310

2281  TTTTAGAAATACAATGGATCCAGTTGAAAAAGCTCTTCGTGATGCTAAGATGGACAAAGG  2340
 311    F  R  N  T  M  D  P  V  E  K  A  L  R  D  A  K  M  D  K  G   330

2341  ACAAATTCACGATATTGTTCTTGTTGGAGGATCAACCCGTATTCCAAAAGTTCAAAAACT  2400
 331    Q  I  H  D  I  V  L  V  G  G  S  T  R  I  P  K  V  Q  K  L   350

2401  TCTTTCTGATTTCTTCTCTGGAAAGGAACTTAACAAATCTATCAATCCTGATGAAGCTGT  2460
 351    L  S  D  F  F  S  G  K  E  L  N  K  S  I  N  P  D  E  A  V   370

2461  AGCTTATGGTGCTGCTGTTCAAGCTGCTATTCTTTCAGGAGACAAATCTGAAGCTGTCCA  2520
 371    A  Y  G  A  A  V  Q  A  A  I  L  S  G  D  K  S  E  A  V  Q   390

2521  AGATTTACTTCTTCTTGATGTCGCTCCACTTTCTCTTGGAATTGAAACTGCTGGTGGTGT  2580
 391    D  L  L  L  L  D  V  A  P  L  S  L  G  I  E  T  A  G  G  V   410

2581  TATGACAGCTCTTATTAAGAGAAACACTACTATTCCAACTAAGACTTCTCAAACTTTCAC  2640
 411    M  T  A  L  I  K  R  N  T  T  I  P  T  K  T  S  Q  T  F  T   430

2641  CACCTATGCTGATAATCAACCAGGTGTCTTGATTCAAGTTTATGAAGGAGAGCGTGCTAT  2700
 431    T  Y  A  D  N  Q  P  G  V  L  I  Q  V  Y  E  G  E  R  A  M   450

2701  GACTAAAGATAATAATCTTCTTGGAAAATTCGAACTTTCTGGAATTCCTCCAGCTCCACG  2760
 451    T  K  D  N  N  L  L  G  K  F  E  L  S  G  I  P  P  A  P  R   470
```

```
2761  TGGTGTTCCACAAATTGAAGTTACTTTTGATATTGATGCTAACGGTATTCTTAATGTATC  2820
 471   G  V  P  Q  I  E  V  T  F  D  I  D  A  N  G  I  L  N  V  S   490
              .        .        .        .        .        .

2821  TGCTCAAGATAAATCTACCGGAAAGCAAAACAAAATTACTATTACCAACGACAAAGGACG  2880
 491   A  Q  D  K  S  T  G  K  Q  N  K  I  T  I  T  N  D  K  G  R   510
              .        .        .        .        .        .

2881  TCTTTCTAAGGAAGAAATTGAACGTATGGTTAATGATGCTGAAAAGTACAAGGCTGATGA  2940
 511   L  S  K  E  E  I  E  R  M  V  N  D  A  E  K  Y  K  A  D  D   530
              .        .        .        .        .        .

2941  TGAAAAACAACGTGATCGTGTAGCTGCTAAGAATGGCCTTGAATCATACTGCTTCAACAT  3000
 531   E  K  Q  R  D  R  V  A  A  K  N  G  L  E  S  Y  C  F  N  M   550
              .        .        .        .        .        .

3001  GAAACAAACTCTTGAAGACGAAAAGGTTAAGGATAAGATTCCAGCTGATGATGCCAAGAA  3060
 551   K  Q  T  L  E  D  E  K  V  K  D  K  I  P  A  D  D  A  K  K   570
              .        .        .        .        .        .

3061  AGTTCTTGATAAATGTGATGAAGTTCTTAAATGGCTTGACAGCAACCAATCTGCTGAAAA  3120
 571   V  L  D  K  C  D  E  V  L  K  W  L  D  S  N  Q  S  A  E  K   590
              .        .        .        .        .        .

3121  GGAAGAATTTGAAGACAGACAAAAGGAACTTGAAGGTATTTGCAATCCAATcATCACCAA  3180
 591   E  E  F  E  D  R  Q  K  E  L  E  G  I  C  N  P  I  I  T  K   610
              .        .        .        .        .        .

3181  GATGTATGGAGCTGCCGGAGGTCCACCAGGAGGTATGCCAGGTGGTGCCCCAGGAGGTGC  3240
 611   M  Y  G  A  A  G  G  P  P  G  G  M  P  G  G  A  P  G  G  A   630
              .        .        .        .        .        .

3241  ACCAGGAGGTGAAGGTTCTGGACCAACAATTGAAGAAGTTGACTAATTATAATTATTCTT  3300
 631   P  G  G  E  G  S  G  P  T  I  E  E  V  D  *                645
              .        .        .        .        .        .

3301  CAGTAGATCTCCATTTTGACTGATTTTATAATTGTCTTCATTTCTTATTTTTAACTGTTA  3360
              .        .        .        .        .        .

3361  TATATAAATGTAATAAAATAAATTACAAGATTGTTTAATTTTCTAATATTTTATTAAGTA  3420
              .        .        .        .        .        .

3421  TATAAAGACTGTACAATTTTAAGGATATTATTTATAAACACACTAAACGAGCAATTATAC  3480
              .        .        .        .        .        .

3481  TAAAATGTACTAGCATTAAAGAGCACAATATAAAAATTAATATAAATATTAATAATTCCC  3540
              .        .        .        .        .        .

3541  TGAAATTCGGTATGATAAACATATATATATATTGTGAGATAAAAAAACTGATAAAATGAG  3600
              .        .        .        .        .        .

3601  GTAATAATTAAGAGGTAGGAAAAATGAGGTGTGATAAATTAATGAGAACAATAACAAAAT  3660
              .        .        .        .        .        .

3661  GGAAGTATATAATATATGATAATATAAAAAAAAATAAAATTGATATTATATATGTTAATA  3720
              .        .        .        .        .        .

3721  AAAATAGCTGTGTTTATAATAATATAAATTTTACATTAGAAAAAAAGGTGTATACTGTAC  3780
              .        .        .        .        .        .

3781  ATTTTCACCAGAAAGAGACGCCCGCGCGTAAACTTGTGGTTGGAGAGAGTTTTTAGAATT  3840
              .        .        .        .        .        .

3841  TTTTGAATGTTAATATAATGAATTAATATAAAAAAATTTTTCCTAATCATGCTTTCCTTG  3900
              .        .        .        .        .        .

3901  TAATAAATGAAGATTAAATGTTAAAGCATCATTTGACGAATGACCACATCGATTGCAGTT  3960
              .        .        .        .        .        .

3961  GAAAGGATTTTCATATCCATGATATCCACAATGAATATGATATAAAGCCTGATCAGCAAA  4020
              .        .        .        .        .        .

4021  AGCCATTTTACAATGTGTACAAATAAACTTAAATTGATCATCTGAATTTCCAATTTTATT  4080
              .        .        .        .        .        .

4081  TTGTTCTTGAAGATTTGTCAAATGAGCTAATAATCCATTATGTAAAAAATTTATGTTTGC  4140
              .        .        .        .        .        .

4141  GGGTTCTCTTGTTGGGATGGGATTAGGGAGTGTAACACTTCCCATTTGAGGAATTGGTTG  4200
              .        .        .        .        .        .

4201  ATGGATAATTTGTGATGAATTATTATTCCCATCTTCTCTTGAAGAAATATTTAAATCATT  4260
              .        .        .        .        .        .

4261  TTTTGAACCTGGTGTGCTTGCAGACCAATTATCTTCCCCTCCACTTGAATTCCCTTCAAT  4320
              .        .        .        .        .        .
```

```
4321  TTCTGGTGATGATTTCCCTTGTAAACGTTGTGATATTTCATCTAATATCATACCTTTTTT  4380
4381  CCTTTTAGCTCCAGCTATACCATCACGAGAGCTACTAATGCTATCATTAGTCTCATCTCC  4440
4441  TGACATCTTTCCAGAATGTAATGGTGAAACATTTGACTCACCTGAAGCAGAATCAACATC  4500
4501  TGGTGAATTTGTCATATGCTCATCATCTCCTTCACTACCAcTACGTTGTAATCTTTTTAG  4560
4561  TGGAGGTAATGGAGGCATTTCAACTTTTAACGAAAAATTATTTTGTCCTAATTGATTATT  4620
4621  AGATAGAGAACTTAAATTAAATGATGAATTTGTTGATTGTTGATTGGATAAAAAATGTTG  4680
4681  TATATTATGTTTTACAGCATCTTCCTTTGAAAAAGGAACATAATCACACAATTTACATGG  4740
4741  AGTATTTGCATCAATAATACTTTGTACAGAATTAGCTTGTTGAATTTGATTATTAAGTGA  4800
4801  ATTTAGTCTTAATAAAGCATTCATATCTAAATTTTGTTGATTAAATAATAATTGTGGATT  4860
4861  TGATGAATTCATAATATTTGGTAACATAAAACTATTAGCTAAAGATGTTGAAATAGAATT  4920
4921  ACTTATTAATGACATTTGCAGCAGAAAGTGCATAGTCGGCAAGAGCAGCATATGATTCTG  4980
4981  ATAATGATCTTGATACATCATTTGkTGATGCATTAGGTGAATCACCAAAATTTTCTAGAG  5040
5041  C  5041
```

Fig 3.6 Nucleotide sequence of 5.04 kb of the insert of clone 5.1 from the genomic library of *P. trichosuri*. This includes the entire coding sequence of the gene for PtHsp70, the 5' upstream region and the 3' downstream region.
The start codon is at position 1309, the stop codon at position 3284. There is one intron from position 1409-1451. The HSE elements in the 5' UTR are underlined; the element showing 100% homology is highlighted in yellow. Translation of the coding sequence is shown in blue with the signature motifs [Prosite] for Hsp70 protein highlighted in grey.

Table: 3.3 Comparison of the protein sequence of Hsp70 of *P. trichosuri* with the Hsp70 protein sequences of other nematode in the GenBank database.

P. trichosuri V	GenPept acc. No.	% Identity	% Similarity
Dirofilaria immitis	AAA28298	89.5	93.0
Heterodera glycines	AAG47839	89.1	91.1
Setaria digitata	AAD13154	88.0	91.6
Wuchereria bancrofti	AAF66987	87.9	91.3
Brugia malayi	AAC17926	87.9	91.6
Brugia pahangi	AAA27857	87.7	92.2
Caenorhabditis elegans	AAA28078	86.5	90.3
Trichinella britovi	CAA73574	81.9	87.7
Onchocerca volvulus aa 1-95	AAB30260	89.5	91.6
Onchocerca volvulus aa 244-565	AAA29417	92.6	94.7

Discussion:

The heat shock proteins are among the most conserved throughout evolution and the *Hsp70* family is the most conserved of the heat shock genes [Gupta and Golding, 1993]. The *Hsp70* multigene family was originally defined by the size of the gene product and later by significant sequence similarities between genes and to even a greater extent, proteins; they are usually classified into four groups which correspond to their cellular location. All eukaryotic organisms investigated so far have several *Hsp70* genes; in some organisms there are groups of two or three closely linked genes, e.g. the MHC class III linked group of HSP70-1, HSP70-2 and HSP70-HOM in human, rat, mouse [Günther and Walter, 1994]. In *Drosophila* five nearly identical *Hsp70*s are clustered in two groups; in *C. elegans* all six genes characterized so far are found dispersed throughout the genome [Snutch *et al.*, 1988], in *Setaria digitata* two *Hsp70* genes are arranged in tandem [Jayasena *et al.*, 1999]. The number and location of *Hsp70* genes in *P. trichosuri* has not been investigated, however, the pattern of restriction digest fragments intimated a second restriction site for both *EcoR I* and *Hind III* that could be consistent with two closely linked genes in the same clone. The heat inducible *Hsp* genes of eukaryotes have been considered unique in their relative lack of introns. *Hsp70* genes in *C. elegans* possess introns as do the *Hsp70* genes of other nematodes, where genomic DNA has been sequenced: *Brugia malayi* and *Setaria digitata* both have nine introns. *P. trichosuri* therefore appears unusual in having only one small intron.

The newly determined sequence for Pt *Hsp70* was not used for formal phylogenetic studies but it is still of some interest to compare sequences. The extremely high degree of homology shows the high degree of functional constraint on this gene. Detailed comparisons and alignments of *Hsp70* sequences have been carried out using a large number of sequences from a diverse range of organisms: archea, bacteria, organelles, protists and eukaryotes [Karlin and Brocchier, 1998]. This has culminated in a global alignment in which there are a number of perfectly conserved amino acid positions throughout all these sequences; the signature motifs have already been mentioned. Because of this high level of conservation *Hsp70*s are considered useful candidates for studying ancient evolutionary relationships, e.g. that between animals and fungi [Borchellini *et al.*, 1998], rather than more recent speciation and sub-speciation.

*Hsp70*s have not been used in two relatively recent phylogenetic frameworks for the Nematoda both of which use the small subunit rRNA genes [Blaxter *et al.*, 1998, Bürglin *et al.*, 1998]. When *Hsp70* was used to investigate the phylogenetic relationships of *Trichinella britovi* it determined the somewhat self-evident fact that it was most closely related to the two other nematodes used [Vayssier *et al.*, 1998]. *Hsp* sequences, however, have proved useful in investigating *Babesia* and the Apicomplexa by increasing the number of parsimony informative characters available due to high level of homology across the entire molecule [Ruef *et al.*, 2000].

It is always of some value to add another *Hsp70* sequence to the many in the database to use for comparative purposes. The conserved *Hsp70* sequences do offer an alternative method of analysing phylogenetic relationships using a functional protein rather than an RNA sequence and the relationship of *P. trichosuri* to other nematodes would be of interest for two reasons. Firstly, because this parasite is associated with Australia's unusual marsupial fauna and consequently was separated from other nematode species at the time of the separation of eutherian from marsupial mammals, currently estimated at around 135 million years ago [with a range of 71-170 million years ago, Hope *et al.* 1990]; also because of its possible use as a model parasitic organism, as discussed in Chapter VI, where the relationship is fundamental for comparative purposes.

Chapter IV Production of *P. trichosuri* Hsp70 promoter construct and microinjection experiments

Introduction:

One of the main objectives of this study was to isolate a strong, native *P. trichosuri* promoter, which would cause expression of transgenes in transfected *P. trichosuri*. *P. trichosuri* have been microinjected with reporter genes under the control of the *C. elegans* promoters col13, col19 and a laminen gene but reporter gene expression has not been observed with any of these constructs [Gruenberg *et al.*, 1999]. Although in *C. elegans*, species specific sequences have not been found to be necessary for transgene expression [Stinchcomb *et al.*, 1985, Grant *et al.*, 1992], this might not be the case in *P. trichosuri* and the promoter sequence from the *Hsp70* gene that had just been determined might overcome any inter-specific promoter differences that could exist.

Heat shock promoters in general, including *Hsp70* promoters, have been used to control expression of transgenes in many organisms and are particularly useful for controlled induction of expression of genes with some stressor, e.g. in investigation of development in animals [Wheeler *et al.*, 2000, Dietrich *et al.*, 2000, abs.] and in the investigation of gene function in transgenic plants [Schroda *et al.*, 2000]. In *C. elegans* the highly inducible hsp16 promoter in strain PC72 has been used to investigate the heat shock response of the animal and the function of many other genes [Stringham *et al.*, 1992, Link *et al.*, 1999]. A transgenic strain, CB4027, with a heterologous Drosophila *Hsp70* promoter has also been used; another role for this strain is as a bio monitor of pollution in water samples in the "Stressed Worm" project [Jones *et al.*, 1996, Mutawakil *et al.*, 1997]. The homologous *Hsp70* promoter has been used but less widely [e.g. Junkersdorf *et al.*, 2000, abs.].

In most organisms control of *Hsp70* genes is at the level of transcription and this has been extensively studied; there is considerable conservation of the mechanism of transcription, as shown by the ability of *Drosophila* heat shock consensus or regulatory elements [HSE] to function in mammalian cells [Pelham, 1985]. When stress occurs heat shock transcription factor [HSF], which is present in the nucleus and cytoplasm in

a monomeric form, trimerizes and binds to its specific recognition sequence, the heat shock element [HSE] [Morimoto, 1993, 1998]. This HSE sequence is found in the 5' untranslated region of all *Hsp70*s investigated so far. A mechanism of transcriptional activation, studied in *Drosophila,* shows that there is a region of the *Hsp70* promoter, delimited by a GAGA binding factor, which is constitutively hypersensitive to DNase1 digestion. The TATA binding protein is constitutively bound to the TATA box and RNA polymerase II starts transcription then pauses approximately 40 base pairs downstream from the transcription start site [Lis, 1998]. When the cell is stressed HSF binds to the DNase1 hypersensitive site reversing the promoter pausing and stimulating re-initiation of transcription and elongation. There is also a chromatin remodelling complex that acts with the GAGA binding factor to slide nucleosomes over the DNA causing a nucleosome-free region to which the HSF can now bind thus stimulating transcription [Aalfs and Kingston, 2000].

Investigation of transcriptional control in *C. elegans* has shown that, of the genes so far studied, most have been controlled by local flanking regions of no more than several kilobases. In the majority of cases these regions have been upstream of a transcriptional start or splice site [McGhee and Krause, 1997]. The *Hsp70* promoter of nearly all vertebrate *Hsp70* genes consists of a relatively short upstream sequence containing, as its most characteristic feature, a number of HSE sequences. These reasons, in addition to the observation of several sequences known to be involved in transcriptional activation, indicated that the 5' region upstream of the coding DNA of this gene could be a useful promoter sequence which might advance us in our attempts to transfect *P. trichosuri*.

Methods:

5.1] Preparation of the promoter fragment and microinjection expression plasmid

The sequence information obtained in Chapter III.5, Fig 3.7 was used to design primers: Pt-prm1 was designed from nucleotide position 1-21 and Pt-prm2 from 1308-1287 with nucleotides 1309, 10, 11 being the ATG start codon. Pt-prm1, the sense primer, was designed with a *NotI* site at the 5' end and Pt-prm2, the antisense primer, had an *AscI* site at the 3' end to enable the fragment to be cloned into the promoter site of an existing vector - pWC33. Primer sequences are shown in Table 3.1. Pt-prm1 and Pt-prm2 were used to amplify the 1.3 kb of 5' sequence upstream from the coding DNA start from clone 5.1 using the Expand High Fidelity PCR System [Roche] under the following conditions: Mg^{2+} concentration of 1.5mM, primers at a final concentration of 0.2 µM, T°anneal =55°C and 29 cycles of amplification. The product of several reactions was purified with High Pure PCR Product Purification Kit [Roche] and samples of 5µg and 10 µg were digested with 40 units *AscI* [NEB] and 40 units Not1 [NEB] in a two-step digestion process. The digestion products were again purified on a High Pure column, quantified approximately by comparison with a mass standard on an agarose gel, then 200ng were used in the ligation reaction as described below.

Plasmid pWC33 outlined in Fig 4.1 was derived from plasmid pPD95 obtained from A. Fire, Carnegie Institute, Baltimore [Fire *et al.*, 1990]. It has been used in our lab with a number of different gene promoters to direct expression of the reporter genes green fluorescent protein from jellyfish [GFP] and β galactosidase [*lacZ*] in transfection experiments with both *C. elegans* and *P. trichosuri*.

5ng of plasmid pWC33 containing a cBAT(2) promoter was transformed into XL2 competent cells by electroporation [GenePulser II, BioRad]. Transformants were grown on LB Amp plates, amplified in liquid culture and plasmid DNA was prepared using a QIAGEN Midiprep Kit according to the manufacturer's instructions. Plasmid DNA was purified and 15µg was digested with 40u *AscI* [NEB] and 30u *NotI* [NEB]. After checking that digestion appeared complete, the entire products of digestion were run on a preparative 1% agarose gel, the piece of plasmid minus promoter insert, of

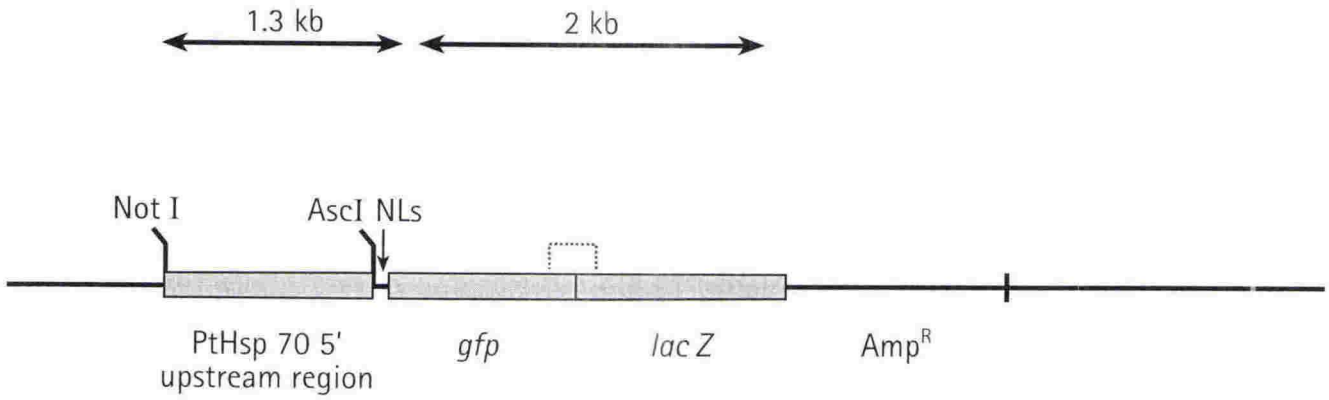


Fig 4.1 Structure of the insert of plasmid pPtHsp70-prm used for the transfection of *C. elegans* and *P. trichosuri*.

The putative promoter from the *Hsp70* gene from *P. trichosuri*, consisting of 1.3kb from the region upstream of the start codon, was cloned into the *NotI* / *AscI* site of an existing vector in frame with the reporter gene for the fusion protein GFP/β galactosidase.

NLS - nuclear localization signal
gfp - gene for green fluorescent protein
lacZ - β galactosidase gene
Amp^R - ampicillin resistance gene

approximately 7.5 kb, was cut from the gel, see Fig 4.3B, and the DNA extracted using the Concert Gel Rapid Extraction System [Life Technologies]. This was then used in ligation reactions with the Pt-*Hsp70* promoter fragment.

Approximately 200ng of insert Pt-*Hsp70* promoter DNA, 50ng of vector DNA, 400 units of T4-DNA Ligase [NEB] with 1µl x10 Ligase Buffer [NEB], were incubated at 16°C overnight. Transformation reactions were carried out as follows: 1] 3µl of the ligation mix above was transformed directly into 50µl XL2 electrocompetent cells, 2] a control of ligation reaction with vector DNA but no insert, 3] ligation reaction with vector DNA but no insert or ligase, to show vector is completely cut and will not self re-anneal, 4] a positive control transforming old complete vector pWC33 cBAT(2) into XL2 cells and 5] a negative control transforming XL2 cells with no vector or insert DNA, see Table 4.1. Six individual transformed colonies from the test reaction were grown in 10ml of LB amp broth and DNA from two of these was prepared, again using the QIAGEN Midiprep Kit. As the new promoter of Pt*Hsp70*, was approximately the same size as the cBAT(2) promoter, verification of the presence of the new promoter in the plasmid was done by PCR using *P. trichosuri* promoter specific primers. DNA was re-precipitated in potassium acetate buffer [0.1M, pH7.0] containing 70% v/v ethanol then re-suspended at a concentration of 80ng/ul in 1 µl of MilliQ water for use in microinjection studies as follows.

5.2] Microinjection of *C. elegans*

All microinjections and screenings for transgenic progeny were performed by Dr Stephen Skinner, a colleague at AgResearch, Wallaceville Animal Research Centre, Upper Hutt, Wellington, New Zealand.

Microinjections were carried out using an inverted microscope with Normarski interference optics, a micromanipulator and microinjector. In order to test the construct, pPtHsp70-prm, for promoter function the plasmid was first injected into the model nematode *C. elegans*, Fig 4.2. These experiments have been described by Skinner *et al.*, 2001. Briefly, *C. elegans* (Bristol N2 strain) were grown on NGM plates [Brenner, 1974] with OP50 or HB101 strains of *E. coli* as a food source. Adult hermaphrodites

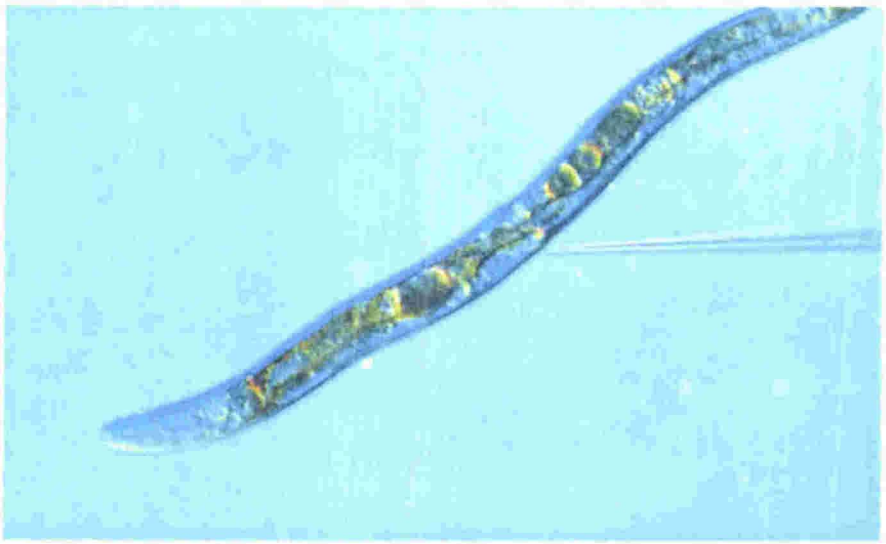


Fig 4.2 Microinjection of a nematode [*C. elegans*] showing relative size of needle and position of gonad. [magnification X100]

were immobilized on dry agar pads under halocarbon oil and plasmid DNA injected into the distal arm of the ovary. To enable selection they were co-injected with pPtHsp70-prm [80ng/ul] and pRF4 [80ng/ul][Mello *et al.*, 1991]; the plasmid pRF4 contains an allele of the *rol-6* gene that confers a rolling phenotype on transgenic offspring. After injection worms were cultured on new NGM/*E. coli* plates. Progeny of injected animals that displayed roller behaviour were selected and sub cultured onto fresh plates, allowed to reach maturity and lay eggs before being viewed by ultraviolet illumination. Animals showing fluorescence were cultured for a further five days then progeny were removed from the plate and stained for *lacZ* expression using a β-galactosidase reporter gene staining kit [Sigma, St. Louis, Mo, USA], [Fire *et al.*, 1990]

5.3] Microinjection of *P. trichosuri*

5.3.1 Free-living *P. trichosuri*

Free-living adult females from laboratory cultures [Gruenberg and Bisset, 1997. Skinner, personal communication] were microinjected with pPtHsp70-prm [80ng/ul] returned to fresh plates with males, mating presumed to occur and the progeny allowed to develop through several generations before being removed for viewing under UV illumination and/or stained for *lacZ* expression as above.

5.3.2 Parasitic *P. trichosuri*

Adult parasitic worms were removed from the small intestine of the possum as described in Chapter II.1.5 and placed in NCTC medium plus antibiotics and antimycotics until microinjected with pPtHsp70. Both males and females were injected, see Fig 4.3. Injected worms were placed in medium at 37°C, 5%CO_2 overnight with un-injected worms of the opposite sex; worms entangling each other were observed and mating was assumed to take place. Eggs and larvae were removed from the medium to NGM plates with a lawn of *E. coli* [HB101 strain] at 12-24 hours post-injection and allowed to develop through several free-living generations for up to 12 days. Progeny were screened for presence of the transgene by viewing under UV illumination and/or staining for *lacZ* expression.

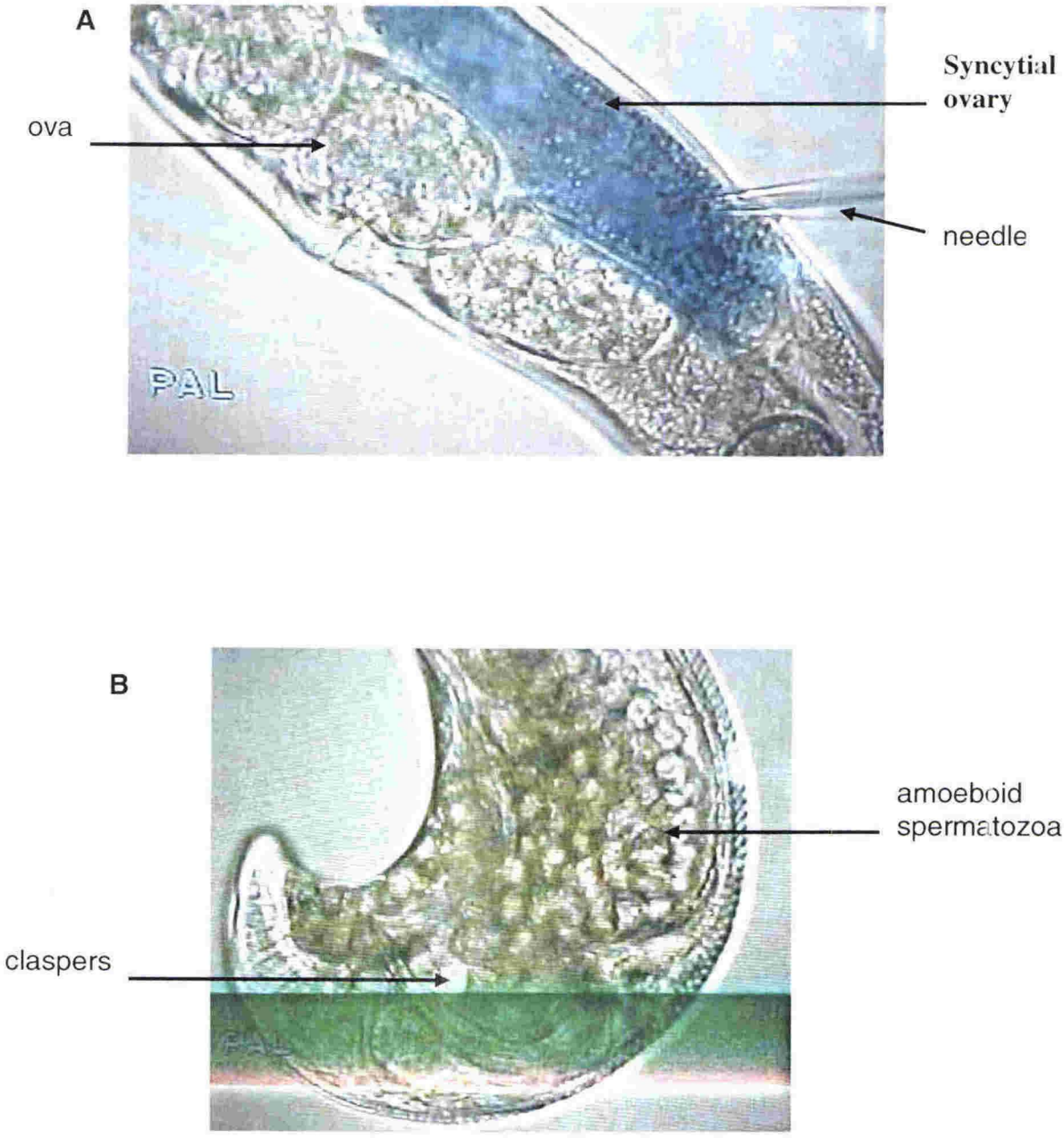


Fig 4.3 Sites of microinjection in parasitic *P. trichosuri*. [magnification X200]

A. An ovary injected with blue dye to show site of microinjection of exogenous DNA. Mature ova in the oviduct can also be seen.

B. Spermatozoa, showing their rounded shape, can be seen in the testis.

Results:

A DNA fragment upstream of an ATG initiator codon in the newly sequenced Pt*Hsp70* gene was amplified, purified and inserted into a plasmid vector before the reporter gene fusion *gfp:lacZ*. This construct was injected, first into *C. elegans* to test for promoter function and then into parasitic and free-living adults of *P. trichosuri*. Progeny of injected adults were screened for expression of the transgene by looking for evidence of green fluorescence or staining for the presence of β galactosidase activity.

5.1] Preparation of the promoter fragment and microinjection expression plasmid

Primers Pt-prm1 and Pt-prm2 were used to amplify the whole fragment of the Pt*Hsp70* promoter region upstream from the coding DNA for which the sequence was known. The PCR reaction gave a single product of the expected size, 1.3 kb, shown in Fig 4.4, which was digested with restriction enzymes *NotI* and *AscI* to create compatible ends for cloning into the vector.

DNA from plasmid pWC33 was digested with *AscI* then *NotI*; Fig 4.5 shows that digestion of the vector was complete and the existing cBAT(2) promoter was fully excised. Aliquots of the doubly digested vector were separated on a gel and after gel extraction there was no evidence of any of the original promoter remaining; all aliquots proved suitable for making the new plasmid, Fig 4.5C. Ligation of the *Hsp70* promoter region into the vector was carried out and the resulting product transformed into XL2 cells; results of the transformation reaction are shown in Table 4.1. The resulting colony numbers are not large but the difference between the vector-insert reaction and the controls is sufficiently clear to believe that these colonies contain a re-circularized plasmid having the new promoter, designated pPtHsp70-prm. The presence of this promoter in plasmid pPtHsp70 was confirmed by PCR of all samples using the *P. trichosuri* specific primers Pt-prm1 and Pt-prm2 as shown in Fig 4.6. The plasmid DNA was re-precipitated then re-suspended in neutral potassium acetate buffer and used at a concentration of 80ng/ul in the following microinjection studies.

Table 4.1 Ligation and transformation reactions in the preparation of plasmid

Ligation Reagents	Tube No.				
	1	2	3	4	5
Positive control - plasmid pwc 33	-	-	-	✓	-
Vector DNA	✓	✓	✓	-	-
Insert DNA	✓	-	-	-	-
X10 Ligase buffer	✓	✓	✓	-	-
T4-DNA ligase	✓	✓	-	-	-
Transformation Reaction					
50 µl x L2 cells	✓	✓	✓	✓	✓
No. of colonies grown - Plate 1	65	0	0	70	0
No. of colonies grown - Plate 2	55	0	0		

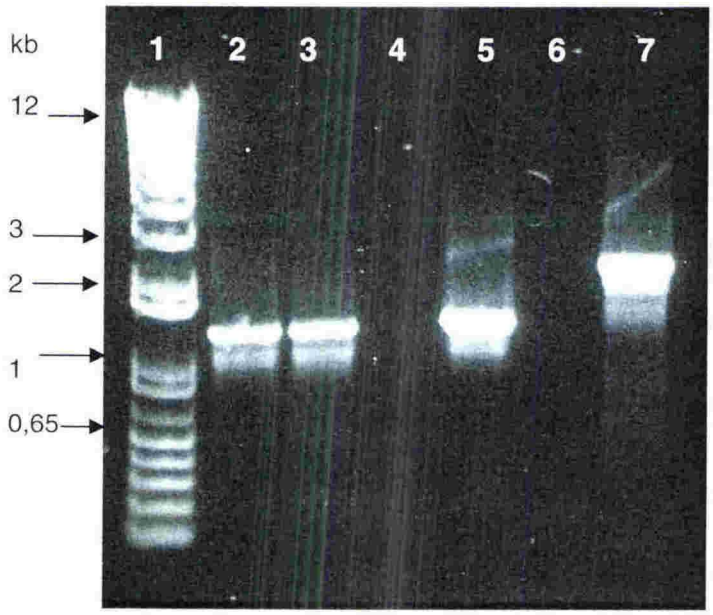


Fig 4.4 Preparation of the promoter fragment from the *P. trichosuri Hsp70* gene.

The putative promoter of the *Hsp70* gene was amplified from the *P. trichosuri* genomic clone 5.1 using with primer pair Pt-prm1/Pt-prm 2. A product of the expected size, 1.3kb, is seen in lanes 2, 3, and 5. A positive control from the coding part of the gene is shown in lane 7 [Pt-J→Pt-L, expected product size of 2.2 kb] and a negative control in lane 6.

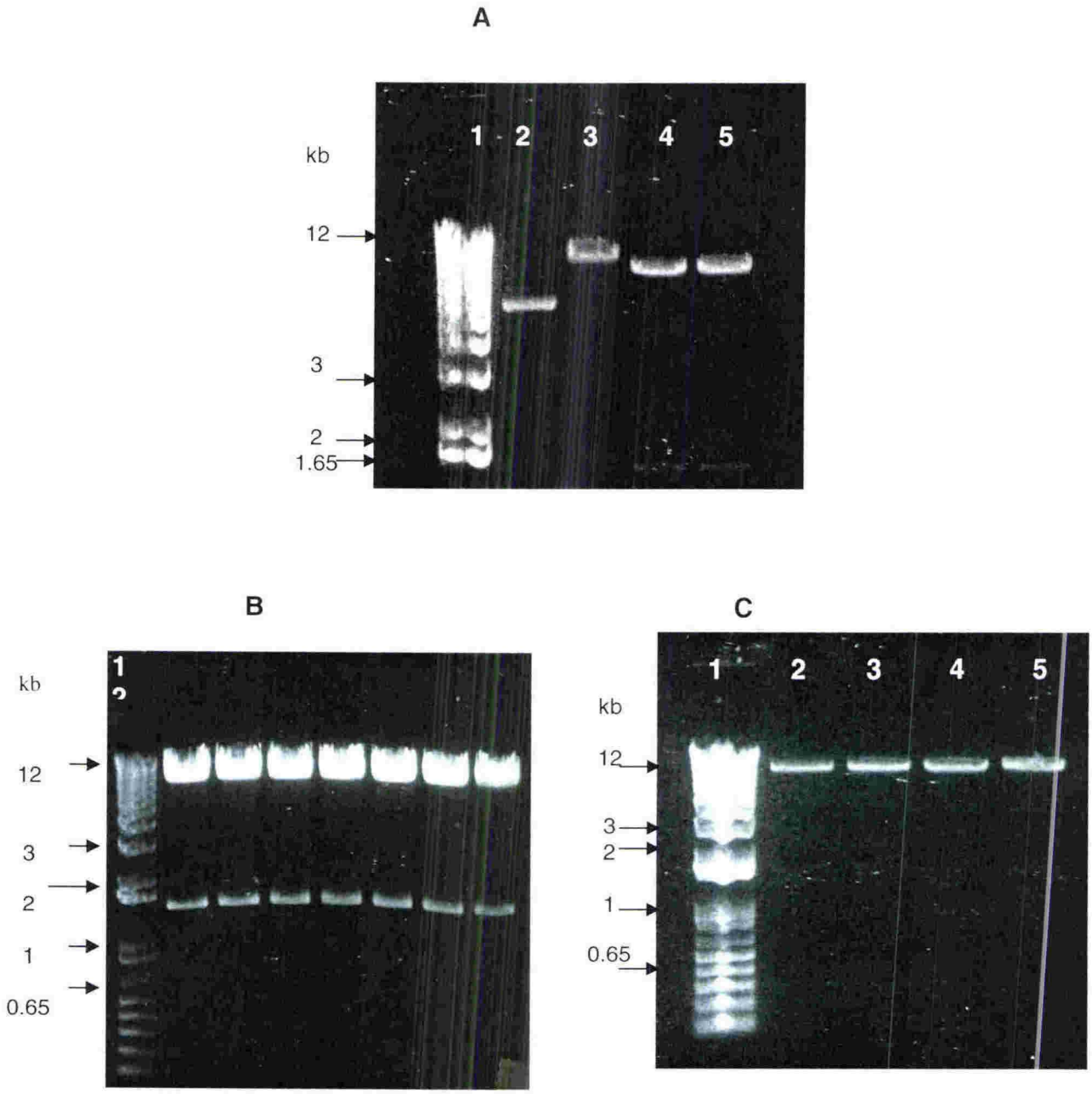


Fig 4.5 Production of construct pPtHsp70-prm from plasmid pWC33

A Precursor plasmid pWC33 is shown uncut [lane 2], digested with *AscI* only [lane 3] and with *AscI* and *NotI* [lanes 4,5].

B: Aliquots of digested plasmid pWC33 prior to purification, showing the excised promoter cBAT(2) fragment.

C: Extracted and purified vector DNA used for cloning the new promoter fragment from *PtHsp70*.

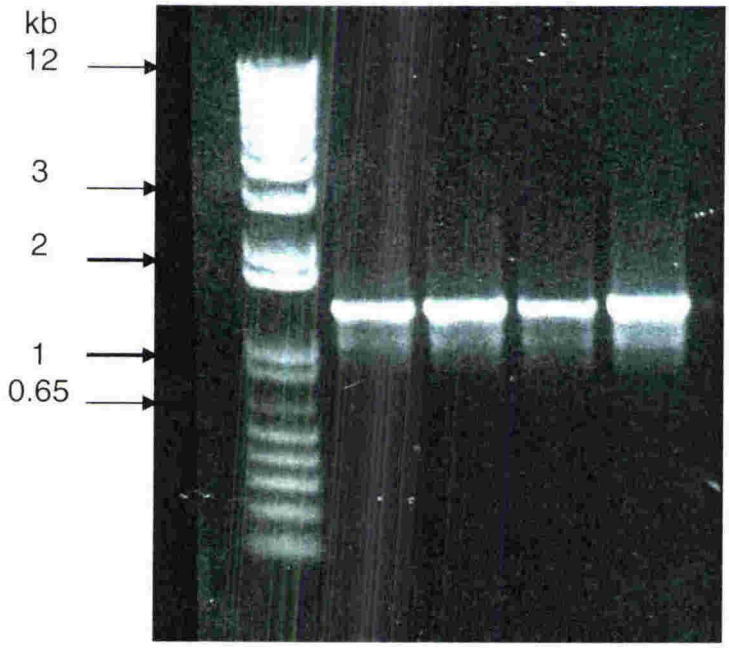


Fig 4.6 Confirmation of the presence of PtHsp70 promoter region in the new plasmid, pPtHsp70-prm.

Preparations of plasmid pPtHsp70-prm were analysed by PCR using the primer pair Pt-prm1/Pt-prm2. The product of expected size, 1.3kb, corresponding to the putative promoter region of the *PtHsp70* gene is present in all preparations.

Microinjection experiments:

As noted on p138 under Methods for Section 5.2, all injections and screening for transgenic progeny were carried out by Dr Stephen Skinner. Dr Skinner also took the photographs of offspring showing expression of the transgene. The concept of microinjecting parasitic forms of *P. trichosuri* was the author's alone and was entirely novel. The author collected the parasitic organisms, as discussed in Chapter II.1.5, and prepared them for microinjection with plasmid pPtHsp70-prm. Plasmid pPtHsp70-prm was prepared by the author. Culturing the post-injection parents and the post-injection removal of eggs and larvae produced to NGM plates was carried out by the author in the initial experiments from which the pictures shown in this section were taken.

5.2] Microinjection of *C. elegans*

Young adult hermaphrodites were injected with either pPtHsp70-prm or this plasmid plus pRF4. The survival of injected worms and the number and viability of their progeny was comparable with transfection experiments using other constructs [Skinner, personal communication].

After three to four days culture about 20% of the progeny of the injected worms appeared to have the roller phenotype. These were sub-cultured and the progeny viewed under UV illumination; green fluorescence was visible behind the pharynx and in the tail in many of the roller animals. Several of these fluorescent animals were stained for β-galactosidase; the stained nematodes were mounted in 60% glycerol, viewed and photographed, as shown in Fig 4.7. The staining is clearly present in the embryo before and after laying, in various stage larvae and the adult. The main area of staining is the gut with strong nuclear localisation. There are clusters of stained nuclear regions in the rear gut and in the region immediately behind the pharynx, Fig 4.8. Some roller phenotype animals were heat shocked by exposing them to a temperature of 33°C for 40mins but no increase of expression of GFP or *lacZ* was seen.

Single worm PCR using the Pt-prm1 and –prm2 primer pair was carried out on individual *C. elegans* with roller phenotype and on an equal number of un-injected

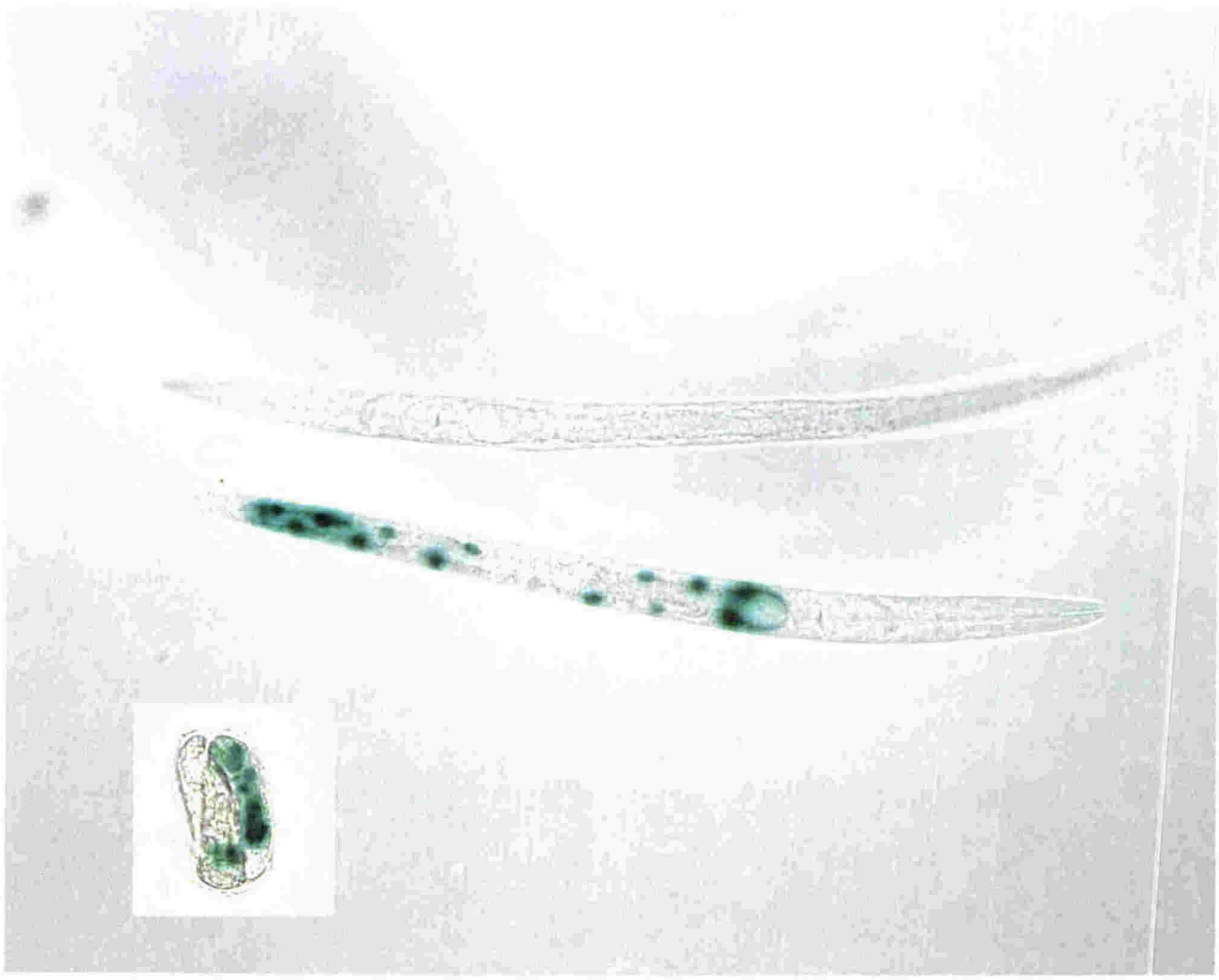
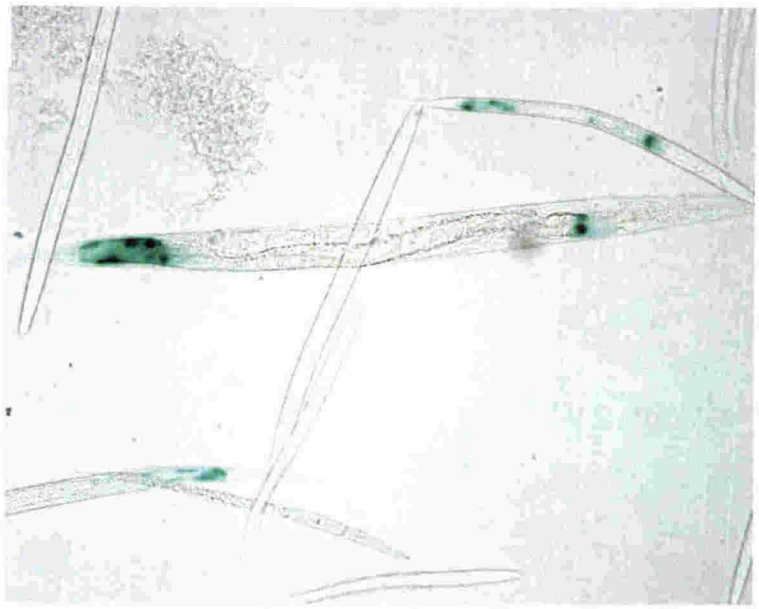


Fig 4.7 Transgenic *C. elegans.*
[magnification X100]
The lower worm is the offspring of an adult microinjected with the construct pPtHsp70-prm and stained for expression of the reporter gene β galactosidase. There is strong blue green staining behind the pharynx and in the tail region. Above is a control worm from an un-injected parent that has been similarly stained.

Insert - β galactosidase expression seen in an embryo

A



B

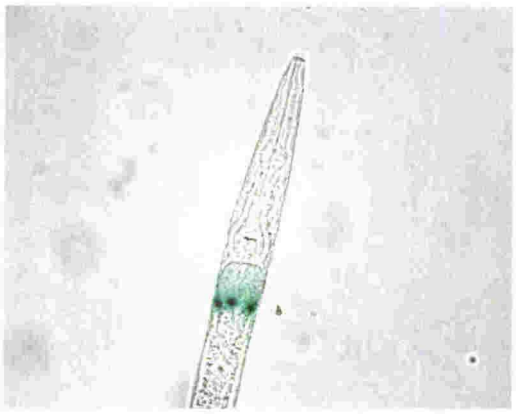


Fig 4.8 Transgenic C. elegans.

A] The pattern of expression of the transgene, β galactosidase, [blue-green staining] is shown in larvae and adults

B] Strong nuclear localization of expression of the transgene, β galactosidase, is seen in the nuclei of cells just posterior to the pharynx.

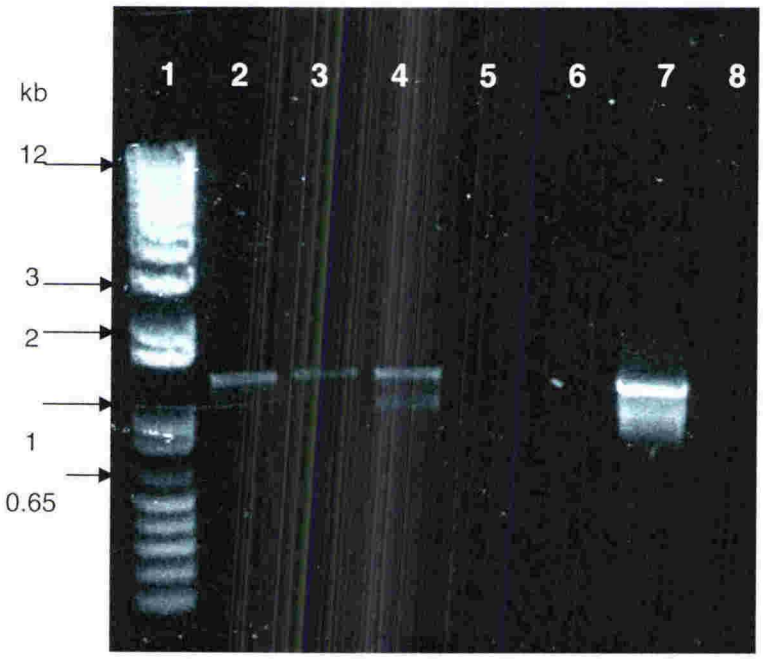


Fig 4.9 PCR analysis of the progeny of injected and non-injected *C. elegans* for the presence of the microinjected construct pPtHsp70-prm, using the primer pair Pt-prm1/Pt-prm2.

Single worms showing the roller phenotype were analysed [lanes 2, 3, 4] along with non-injected control worms [lanes 5, 6]. Plasmid pPtHsp70-prm DNA was used as a positive control [lane 7] and a negative control [lane 8] is included. 1 kb plus molecular weight markers are shown in lane 1.

controls. The results clearly indicate the presence of the Pt*Hsp70* promoter fragment in the progeny of the injected worms and not in the controls, Fig 4.9.

5.3] Microinjection of *P. trichosuri*

5.3.1 Free-living *P. trichosuri*

P. trichosuri have been cultured through the free-living life cycle in our laboratory for several years [Gruenberg and Bisset, 1997, Skinner, unpublished.]; the free living forms are similar to the adults of *C. elegans* but are not hermaphrodite. As described, [ibid] *P. trichosuri* are more prone to damage when injected and far fewer offspring are observed compared with *C. elegans*. When the offspring were screened under UV illumination no fluorescence unequivocally ascribed to GFP could be seen; this may be due to the difficulty posed by the strong endogenous fluorescence in *P. trichosuri*, particularly in the gut. About half of each batch of progeny from injected parents were stained for β-galactosidase expression but on no occasion was staining seen in any worm.

5.3.2 Parasitic *P. trichosuri*

Adults removed from the duodenum and jejunum of infected possums were injected on the same day in either one or occasionally both ovaries. Spermatozoa in *P. trichosuri*, as in many nematodes, are amoeboid and fenestrated and it was presumed that they could take up exogenous DNA similarly to ova, therefore, in addition, adult males were microinjected in the testis. Eggs and larvae appeared in the culture medium at between 12 and 24 hours in relatively small numbers, about 10-70 per culture group of two to five adults. The precise combination of injected female with injected or un-injected males or injected males with un-injected females was immaterial to the numbers of offspring produced or inheritance of the transgene.

The embryos and larvae produced by injected parasitic adults were removed from the medium and allowed to grow and reproduce for up to 12 days. The cultures were sampled at intervals during this time for viewing under UV illumination but no fluorescent nematodes were seen. *P. trichosuri* has a high level of endogenous or autofluorescence and it is possible that this again masked any GFP expression [Billinton

and Knight, 2001]. Again about half of all progeny from each experiment were stained for *lacZ* expression. In two of six experiments there was evidence of blue/green staining in a small number of nematodes, shown in Fig 4.10, Fig 4.11. In the first experiment some expression of the transgene was seen in nine individual worms. The blue/green staining was seen in the pharynx and, in some worms, in the hindgut or tail; it was seen in both males and females, and in some larvae. Staining was less intense than that seen with the same construct in *C. elegans* but was in similar anatomical locations in *P. trichosuri.*

Fig 4.10 **Transgenic *Parastrongyloides trichosuri***

A free-living male *P. trichosuri*, progeny of a parasitic adult microinjected with the construct pPtHsp70-prm, shows staining for the expression of β-galactosidase in the region of the pharynx and hind gut.
[magnification X100]

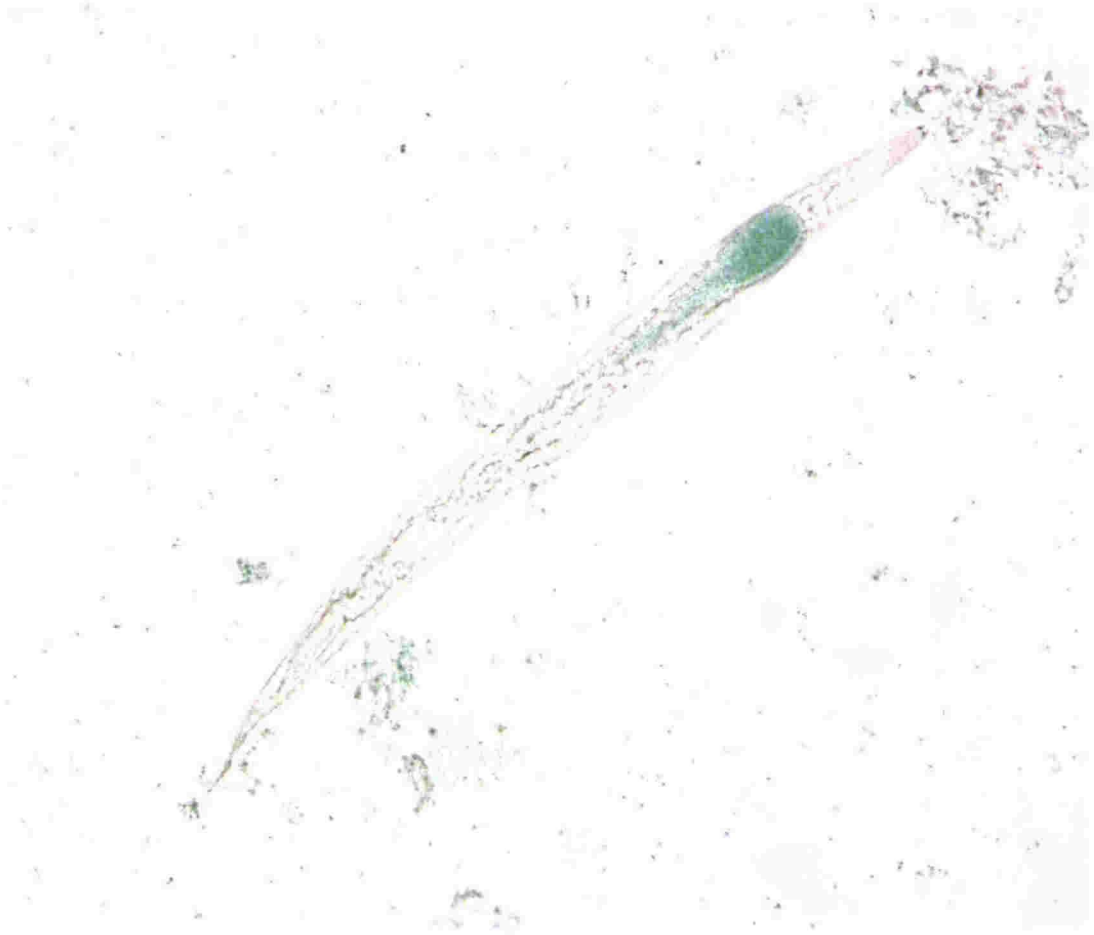


Fig 4.11 Transgenic *P. trichosuri*- second experiment

A free-living female, progeny of a parasitic adult microinjected with the construct pPtHsp70-prm. Transgene expression, shown by the blue-green staining for β-galactosidase, is seen in the region of the pharynx and gut [magnification X100]

Discussion:

As discussed in Chapter III.5, a heat shock consensus sequence was found in the 5' region upstream of the ATG start codon along with several TATA box type sequences. Although the nature or positions of all promoter and enhancer elements for this gene are not known it was decided to use the entire 5' upstream region for which the sequence had been determined to test for promoter function in *C. elegans* and then in *P. trichosuri*. The putative promoter sequence from the cloned *Hsp70* gene from *P. trichosuri* was introduced upstream of a reporter construct consisting of a *gfp:lacZ* fusion gene and this successfully gave rise to gene expression in the model nematode *C. elegans*. Free-living *P. trichosuri* continue to produce few offspring after microinjection despite improvements in injection techniques, and to date there has been no expression of the reporter with the plasmid pPtHsp70 or indeed any of our other plasmids in this form of the parasite.

Parasitic adults are much bigger than free-living adults and are subject to the mechanical strains of life in the small intestine; this, it was believed, could render them more resistant to the trauma of microinjection. Also, parasitic adults had been kept in culture and survived for at least ten days and during that time eggs and larvae had been produced, usually within 24-48 hours and again at irregular intervals thereafter [data not shown]. By far the most important factor in the continued survival of these parasites in culture was preventing infection by bacteria and, especially, fungi in the culture medium. Offspring from injected worms were seen in the culture medium after 12 to 24 hr and fewer were produced in the following days. Therefore it was necessary only to keep the injected parent alive for this length of time and infection consequently became less of a problem. On occasions, female worms with embyronated eggs visible inside them but looking somewhat morbid were simply placed on NGM plates and the offspring emerged from the dead adult. It is now routine to remove eggs and larvae from the culture medium at 24 hr post-injection and to retain the adults until progeny have remained alive and passed through at least one free-living life cycle.

The results indicating successful reporter expression in offspring of injected parasitic worms are exciting. The pattern of staining seen so far in both *C. elegans* and *P. trichosuri* appears to be a "pharynx/tail" pattern of expression which has been seen in *C.*

elegans in a number of transgenic promoter-reporter constructs [Hope, 1991, Krause *et al.*, 1994]. This is considered to be ectopic in a number of cases and here may show little resemblance to the pattern of protein expression normally under control of the *Hsp70* promoter. However, normal physiological expression of *Hsp70* genes is not being investigated, all that is required for the purpose of a transgenic bio control agent is that the regulatory sequences do activate a gene and result in production of the gene product in amounts which are biologically active. Some worms were heat shocked under various conditions, e.g. 37°C for 2 hr or 4 hr. No increase in transgene expression was seen [Skinner, personal communication]. These results imply that successful transfection of *P. trichosuri* with a gene for a product deleterious to the possum is, at least, feasible and as such the main objective of this project has been achieved.

In transformation experiments with *C. elegans* the DNA that is microinjected need not derive from that species nor does it need any specific *C. elegans* sequences in order to be expressed in a transgenic worm [Stinchcomb *et al.*, 1985, Grant *et al.*, 1992]. It would be expected, therefore, that *P. trichosuri* would not require species-specific elements either as is assumed, and inheritance patterns indicate [see next paragraph], that injected DNA forms a similar extra-chromosomal array structure in the cells of *P. trichosuri*. However, to date only the construct using the *P. trichosuri* specific promoter has given rise to expression in transgenic offspring. Whether this is due to *P. trichosuri* specific sequences or just to incremental improvements in microinjection and culturing techniques is not yet known. Promoters used in experiments that have resulted in transgenic nematodes of other species are discussed in Chapter VI.

Work subsequent to the successful expression experiments has shown that the exogenous DNA is inherited by, and present in, the offspring of microinjected parasitic parents at a far higher frequency than that in which expression of the transgene has been observed. PCR of single worms using primers specific for the transgene have shown transfected DNA in about 25% of the offspring, see Fig 4.12 [Skinner, personal communication]. Experiments in which individual injected worms and their offspring have been genotyped using this PCR system have shown that heritability of the transgene in the F_1 is usually about 10% for this construct. Offspring of the transgenic F_1s were cultured then genotyped, between 25% and 100% of the F_2 generation were

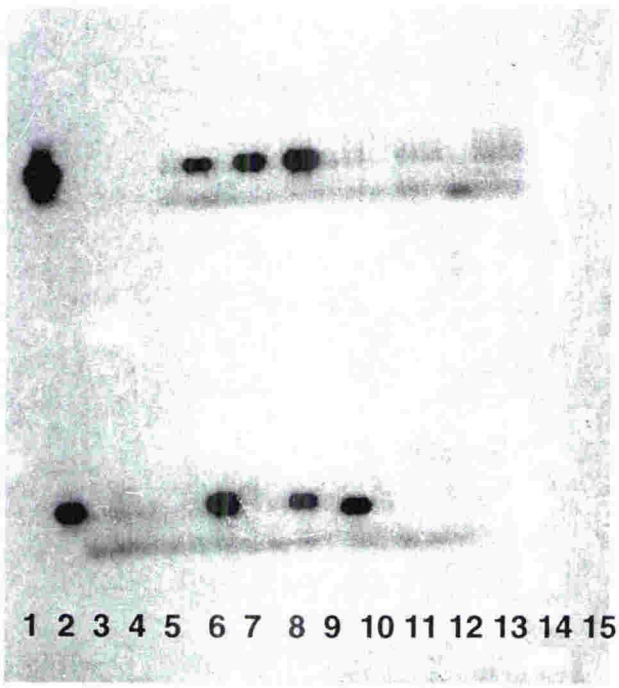


Fig 4.12 PCR analysis of single *P.trichosuri* free-living adult worms

Upper panel: lane 1: *hsp-70* plasmid template as positive control; lanes 2-4: individual free-living adults from cultures established from uninjected parasitic adults (negative controls); lanes 5-15: individual free-living adults from cultures established from injected parasitic adults.

Lower panel: lane 1: blank; lanes 2-15: as for upper panel lanes 5-15 (i.e. at total of 25 individual worms from injected parasitic adult cultures). The PCR amplifies a region between the promoter and the GFP gene in the construct.
[Skinner SJM, with permission.]

transgenic. The percentage was variable, which is consistent with an extra-chromosomal mode of inheritance [Grant, personal communication]. The original observations of transgenesis were on worms that were several generations removed from the injected parents, thus showing that inheritance of the introduced DNA is reasonably stable.

Expression levels continue to be low, fewer than 1% of the progeny positive for the transgene [by PCR] show detectable level of β-galactosidase activity. Achieving reasonably high and consistent levels of expression is, of course, a common problem in all transgenic animals [Brower, 2001, Grosshans, abs. 2000]. Possibly, expression from a non-integrated gene present in an abnormal copy number may be difficult for the animal to regulate but this should hold true for expression in *C. elegans* as in *P. trichosuri* and the frequency of expression was far higher [approaching 20% for the *rol-6* marker] in the former. As mentioned, there may well be sequences other than those in the introduced immediate 5' upstream region that are required for strong expression from this promoter in our worm. The plasmid could be modified to include the short intron found at position 100-143 in *P. trichosuri* instead of, or in addition to the existing intronic sequences. In many of the vectors used for investigation of gene function in *C. elegans* the 3' flanking region from *unc54* has been adequate to ensure good gene expression [Fire *et al.*, 1990]. Possibly *P. trichosuri* requires the use of specific 3' flanking regions and modification of this area to include some specific 3' downstream sequences might enhance gene expression. In *Leishmania infantum Hsp70* gene expression requires a 3' downstream sequence for temperature dependent accumulation of mRNAs of the reporter genes [Quijada *et al.*, 2000]. In human cell lines cloning of an element found in the 5' UTR of human *Hsp70* mRNA increased translation of the reporter gene markedly without concurrent increase in transcription [Vivanus *et al.*, 2001]. The testis-specific *Hsp70* genes in mice and rats require *cis* regulatory elements located in an untranslated sequence of mRNA upstream from the ATG site for adequate expression [Scieglinska *et al.*, 2001].

The *Hsp70* promoter from all model organisms is widely used and the function and mode of action of *Hsp70* genes is so conserved that it is possible that sequences found to enhance the activity of this promoter in other organisms may well be of use in *P. trichosuri* also. Alternatively orthologous promoters for strong housekeeping genes and

secretory promoters that have been shown to function well in *C. elegans* could be found from the *P. trichosuri* genomic library with the aim of improving transgene expression.

Transgenic animal technology is a powerful tool for determining gene function and has other possible practical applications. Considering the widespread use of microinjection and its apparent ease in *C. elegans* it is surprising how rarely other species of transgenic nematode have been successfully created. There have been reports of transgenesis in another member of the same genus, *Caenorhabditis briggsae* [Jan *et al.*, 1997], and in *Panagrellus redivivus*, another free-living soil nematode. Two entomopathogenic parasites, *Heterorhabditis bacteriophora* [Hashmi *et al.*, 1995] and *Steinernema feltiae* [Vellai *et al.*, 1999] have been rendered more robust by transfection with genes for increased heat and drought tolerance, and microinjection of males of *Heterodera glycines* the soybean cyst nematode has produced transgenic offspring in this species also [Opperman and Bird, 1998]. Transient expression has been seen in *Litomosoides sigmodontis* injected adults [Jackstadt *et al.*, 1999] and *Ascaris suum* embryos [Davis *et al.*, 1999] and embryos of *Strongyloides stercoralis* have been produced that show strong expression of DNA which has been incorporated after injection into the parental gonad [Lok and Massey, 2002]. These examples will be considered in Chapter VI.

Chapter V Production of recombinant *P. trichosuri* Hsp-70 protein

Introduction

The immunogenic protein of the original observation has been identified as an Hsp70 protein; the gene for one such protein has been sequenced and an element immediately upstream of the translational start has been used to drive a reporter construct successfully in free-living adult *P. trichosuri.*

In a number of studies where antigenic proteins of parasites have been characterized, antibodies have been made and used to detect the normal localization and possible function of the gene product; studies of the expression of genes in particular tissues or parasite life-cycle stages have been done by *in situ* immunolocalization using these specific antibodies raised to the purified protein [Rothstein and Rajan, 1991, Wu *et al.*, 2000, Bürglin *et al.*, 1998]. There were substantial difficulties in purifying the Hsp70 protein from *P. trichosuri,* these have been discussed in Chapter II, and it was considered unlikely that the individual pure protein could be isolated by normal protein purification procedures in sufficient quantities to produce specific antibodies, or for any other studies.

It was decided, therefore, to produce a recombinant protein from the gene that had been sequenced for the following reasons: firstly, to show that this gene was indeed the gene that corresponded to the original protein. Secondly, production of a recombinant protein would allow the raising of an anti-Pt Hsp70 serum to determine the normal expression pattern and localization of this protein in *P. trichosuri;* this could then be compared with the pattern of expression that had been seen in the transgenic animals. Thirdly, epidemiological and ecological studies of this parasite are continuing and such a protein could provide a consistent and quantifiable antigen to develop an immuno-diagnostic test for the infection status of possums with *P. trichosuri;* a refinement of the original work in which the protein was first identified.

Methods:

5.1] Preparation of the Hsp70 coding sequence from *P. trichosuri* cDNA

Infectious L3 larvae were cultured from possum faeces as described previously [Chapter II.1.4], collected, washed and centrifuged briefly at 500 G. They were ground under liquid nitrogen and RNA was prepared from approximately 1.5ml of ground larval powder using a phenol/guanidine isothiocyanate reagent, Trizol® [Life Technologies], according to the manufacturer's instructions. Briefly, the tissue was homogenized, centrifuged and total RNA was precipitated from the aqueous layer with isopropyl alcohol. The RNA was washed and re-suspended in RNase-free water [water treated with Diethyl pyrocarbonate –DEPC] with added DTT and RNase inhibitors at 40units/µl.

cDNA was reversed transcribed from RNA in the above preparation: 4-5µg total RNA was incubated with 0.5µg Oligo dT_{12-18} [Invitrogen] at 70°C for 10min; a reaction mixture containing 200units of Superscript II™ Reverse Transcriptase [Life Technologies], X5 RT buffer, 0.01M DTT, 0.5mM dNTP mix was then added and the reaction was incubated at 42°C for 2hr. DEPC-water was added to dilute the initial concentration of RNA to about 20ng/µl, the enzyme inactivated at 95°C for 5min and the reaction product used in the following PCR reaction.

Primers Pt-ex1 and Pt-ex2 were designed from the genomic sequence, bracketing the coding sequence of the *Hsp70* gene; the sequences are given in Table 3.1. These were used to amplify the coding sequence of the gene using the Expand High Fidelity PCR System [Roche] under the following reaction conditions: Mg^{2+} concentration of 1.5mM, primers at a final concentration of 400nM, T°anneal = 55°C, 29 cycles of amplification, purified and quantified. The PCR product was used at a concentration of 50ng/µl in the sequencing reactions. The sequencing of the cDNA fragment was carried out as described in Chapter III.5 using primers Pt-: ex1, ex2, C, F, L and Nt. Both forward and reverse strands were sequenced.

5.2] Cloning of the *P. trichosuri* Hsp70 gene into an expression vector

The primers Pt-ex1 and Pt-ex2 were designed with restriction sites for *NdeI* and *NotI* respectively to facilitate precise positioning relative to the promoter of the expression vector and the ribosome binding site. The expression vector BQ 1.1, outlined in Fig 5.1, was provided digested and treated with shrimp alkaline phosphatase at a concentration of about 10ng/µl.

Approximately 1.5µg of *P. trichosuri Hsp70* cDNA was digested with 40 units of *NotI* [NEB] at 37°C overnight; the product was purified and re-digested with 80 units of *NdeI* [NEB] at 37°C for 4hr. About 100ng of this insert DNA was re-precipitated with 30ng of vector DNA, this was re-suspended with T4 Ligase buffer and 400 units of T4-DNA Ligase [NEB] and ligated at 16°C overnight. The enzyme was inactivated and 3µl of the ligation mix was transformed directly into 50µl XL2 electro competent cells; vector only controls were also transformed and grown overnight.

Cultures from six individual transformant colonies were grown in 10ml of LB amp broth and plasmid DNA was prepared from these using the Wizard Plus Miniprep system [Promega] according to the manufacturer's instructions. Bacterial cells from 5ml of overnight culture were lysed and the plasmid DNA removed from the lysate by binding to the Miniprep resin using a vacuum manifold. The DNA was eluted from the resin with warm TE buffer. An aliquot of this DNA [unquantified] was digested with 40 and 80 units of *NotI* [NEB] and *NdeI* [NEB] respectively and the digestion products run on a gel to confirm that an insert of approximately 2 kb, the expected size of the gene, was present.

5.3] Expression and purification of recombinant protein

Protein expression from the cloned plasmid was induced in a 48 well micro titre plate set up as follows: 50µl of cells from overnight cultures were added to 500µl of LB/Ampicillin medium; this was performed in duplicate for each of six cultures. The cells were grown at 37°C until OD_{600} was approximately 1. Protein expression was

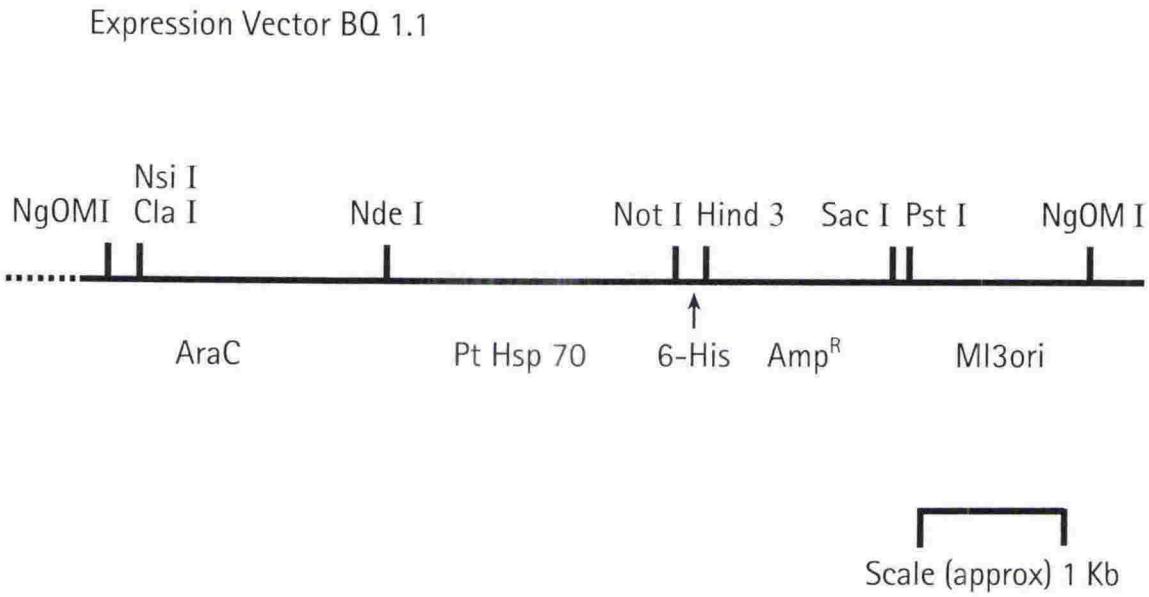


Fig 5.1 Structure of plasmid BQ 1.1 used for expression of recombinant *P. trichosuri* Hsp 70.

The coding sequence of the *Pt Hsp70* gene is cloned into *Nde/Not1* site in frame with a hexahistidine tag at the carboxy terminus. Expression of the gene is under the control of an inducible arabinose promoter.

AraC - arabinose promoter
Amp^R - ampillicin resistance gene
Ml3ori - origin of replication from M13

induced by adding 5µl of 20% arabinose to one of the duplicate samples and the cultures grown for a further 4 hours. The bacteria in each sample were then pelleted, suspended in 50µl of B-Per Bacterial Protein Extraction Reagent [Pierce], mixed vigorously then re-centrifuged at 15,000g in a bench top centrifuge. The supernatant was retained as the soluble protein fraction, the insoluble pellet was re-suspended in a further 50µl of B-Per, 25µl of X5 SDS loading buffer added to all samples which were then boiled for 5min. All samples, soluble and insoluble, induced and un-induced were then loaded onto a 10% acrylamide mini gel in duplicate for electrophoresis.

On the basis of the results of the small-scale induction, one clone BQ1.1-4 was selected for production of the recombinant *P. trichosuri* Hsp70 protein. One litre of LB medium with ampicillin at a concentration of 100µg/ml was inoculated with 45ml of an overnight culture of clone BQ1.1-4. This was grown to a cell density of A_{600} =1, induced with arabinose to a final concentration of 0.2% and grown for a further 4hr. The cell suspension was centrifuged at 2500g for 15min and the cells frozen overnight. Soluble protein was prepared by re-suspending the cells in 20mMTris/20%sucrose buffer, washing, re-suspending in PBS with protease inhibitors then lysing the cells by sonication. Enzymes at a final concentration of 25units/ml of DNase and 8µg/ml of RNase were added and the sonicate was centrifuged at 8000g for 30min.

The supernatant from the sonication was filtered and the protein extracted by immobilized metal affinity chromatography [IMAC] on a Hi-Trap Chelating Column [Pharmacia Biotech] using nickel as the chelating agent. The 1ml column was charged with 0.1M nickel sulphate, equilibrated and washed with a "Start" buffer containing 20mM sodium phosphate and 0.5M NaCl pH7.2. The bound protein was eluted with 20-100mM imidazole solution diluted in "Start" buffer and residual protein and metal ions were removed from the column with 0.05M EDTA. Successive samples of 0.5ml were collected throughout the washing and elution steps and analysed on 10% acrylamide mini gels. Samples with conspicuous amounts of recombinant protein were concentrated by centrifugation at 500g through a 10kD Microsep Concentrator [Filtron] then further purified by FPLC using a Superdex™ 75R separating column [Pharmacia]. The samples of soluble protein from the FPLC column were concentrated and quantified.

Protein present as inclusion bodies was purified as follows: inclusion bodies were washed three times in 25% sucrose with 1% Triton X-100 in PBS then solubilized by sonication in 8M urea in 20mM sodium phosphate, 0.5M NaCl pH7.2 and left on ice for 1hr. The suspension containing the solubilized inclusion body proteins was centrifuged twice at 15000g for 30min, and the his-tagged protein was separated from the supernatant by affinity chromatography using TALON™ cobalt based resin [Clontech]. A buffer containing 50mM sodium phosphate, 20mM Tris-HCl, 100mM NaCl and 8M urea pH8.0 was used to equilibrate and wash the resin; the solubilized inclusion body protein was bound to the resin overnight and the protein eluted with increasing concentrations of imidazole, from 20-100 mM. All samples were analysed on 10% mini gels; samples with reasonable quantities of protein were concentrated in an Amicon stir-cell using a YM 30,000 membrane, the concentrate was further purified by FPLC as described for the soluble protein.

5.4] Immunoblotting of the recombinant protein with possum antibodies

Native larval antigen from Pt L3s was prepared as described in Chapter II.1.4. Samples of this mixture of protein and recombinant protein derived from both the insoluble and the purified soluble fraction were separated on a 5-25% acrylamide gradient gel. The products of electrophoresis were transferred to nitrocellulose and immunoblotted as described in Chapter II.2.1. Four different samples of positive possum serum [serum that had previously been shown to have antibodies] were used at a dilution of 1:1000 in "Blotto", naïve possum serum was used as a negative control. The second antibody, rabbit-anti-total possum Ig was used at a dilution of 1:1000; this was bound by goat-anti-rabbit HRPO conjugate again at 1:1000 and developed with AEC.

5.5] Comparison of recombinant and native 70kD protein

Comparison of the native and recombinant samples of Pt Hsp70 protein was carried out by matrix-assisted laser desorption/ionisation [MALDI] and mass spectrometry [MS] of peptide masses resulting from digestion of the two proteins with trypsin.

5.5.1 Two-dimensional gel electrophoresis of native protein

First dimension isoelectric focussing was carried out using the IPGphor™ [Amersham Pharmacia Biotech] using a narrow pH range immobilized pH gradient of 4-7. Crude antigen was prepared from frozen ground *P. trichosuri* L3 s and total proteins in two samples of 300µl were precipitated with ice-cold acetone at -20°C for 1hr. After drying, the samples were dissolved in 600µl rehydrating and denaturing buffer of 8M urea and 2% w/v CHAPS, 0.5%IPG buffer pH4-7 and a trace of bromophenol blue, they were then centrifuged and applied to two Immobiline DryStrips [pH4-7] according to the manufacture's instructions. The DryStrip holders were placed on the Electrophor unit platform; rehydration was carried out at 30V for 36hr followed by isoelectric focussing according to the voltage protocol: 120V for 1 hr, 500V for 1hr, 1000V for 1 hr, 1000-8000V gradient for 30 min, 8000V for 6.0 hr [Total Volt hours were 52000 to 55904].

Prior to second dimension SDS PAGE, the strips underwent standard equilibration with buffer consisting of 50mM Tris-HCl, 6M urea, 2% SDS, 30%v/v glycerol with the addition of 1% DTT for the first equilibration and 2.5% iodoacetamide for the second equilibration. After equilibration each strip was placed along the top edge of a 10% SDS gel, sealed with 1% agarose and run in Tris-glycine buffer at 25V for 1hr then 150V until dye front reached the bottom of the gel. One gel was stained with Coomassie blue R-250 [microwave assisted], scanned and retained in water; the second was transferred to nitrocellulose. The area on the membrane from 30kD to 94kD was Western blotted using possum serum at 1:500 as first antibody, rabbit-anti-possum total Ig at 1:100 and goat-anti-rabbit-HRPO conjugate at 1:1000. Five protein spots identified by comparison with positive spots on the Western blot were excised from the gel, also a piece of unstained gel was excised as a blank and a piece of the presumed actin spot to act as a positive control.

5.5.2 Tryptic digestion of native and recombinant protein

The samples excised from the acrylamide gel were destained and digested with trypsin [Shevchenko *et al.*,., 1996]. Two samples of recombinant protein were digested in the same manner; Rec5 containing 5µl of the "dilute" soluble fraction at 80µg/ml and rec0.5, a 1:10 dilution containing about 40ng. All these procedures were carried out in a laminar flow hood to attempt to eliminate contamination. Briefly, excised gel spots were macerated and destained in 25mM ammonium bicarbonate [NH_4HCO_3] in 50%

acetonitrile [ACN]. Spots were then dried by centrifugal evaporation in a Speedvac and rehydrated in 25mM NH_4HCO_3 containing 12.5ng/µl trypsin [modified sequencing grade, Roche] and incubated at 37°C for 16 hr.

The resulting peptides were extracted sequentially with 25 mM NH_4HCO_3, 50% ACN/0.5% trifluoroacetic acid [TFA] three times, followed by a final extraction in 100% ACN. The combined extracts were dried in a Speedvac, rinsed with milliQ water, then dried again. Extracts were dissolved in 0.5%TFA then purified using ZipTips C_{18} [Millipore] according to the manufacturer's directions: the tips were wetted with 50% ACN, equilibrated with 0.1%TFA and the peptides bound by repeated aspiration of the samples. Bound peptides were washed with 0.1%TFA then eluted from ZipTips in matrix solution consisting of a saturated solution of α-Cyano-4-hydroxycinnamic acid in 50% ACN/ 0.5% TFA; peptides were spotted directly onto a Maldi sample plate. Calmix 2 [Sequazyme™ Peptide Mass Standards Kit, PerSeptive Biosystems] was spotted between each four samples and used as an external calibration.

5.5.3 Mass fingerprinting of peptides by mass spectrometer

Molecular masses of tryptic peptides from each protein spot were determined on a MALDI-TOF instrument equipped with a nitrogen laser at 337 nm [PerSeptive Biosystems, Voyager mass spectrometer] at Victoria University, [Wellington NZ]. Peptide masses were submitted for protein mass database searching at:

ProFound **[URL:** http://www.proteometrics.com/prowl-cgi/ProFound.exe]. [30/11/2000]

Results:

In order to prepare a recombinant PtHsp70 protein, mRNA was prepared from infectious L3 larvae, this was reverse transcribed and the coding sequence for the gene amplified from the cDNA. This sequence was cloned into an expression vector and expressed in *E. coli*. The reaction of the recombinant protein with antibodies in possum serum was compared with native protein and the two were compared by mass fingerprinting of the products of trypsin digestion by MALDI-TOF.

5.1] Preparation of the Hsp70 coding sequence from *P. trichosuri* cDNA

RNA samples having a concentration of from 0.3-0.5 mg/ml and of acceptable purity [$A_{260/280}$>1.7] were prepared from ground infective larvae and cDNA was reverse transcribed from this RNA. This was used as a template in the PCR reactions of the *Hsp70* gene.

Pt-ex1, the sense primer, was designed with a 5' *AscI/NdeI* site and Pt-ex2, the antisense primer, was designed with a *NotI* site 5' to the last codon, immediately preceding the stop codon at position 3284 to enable the fragment to be cloned in frame into the expression vector BQ1.1. The theoretical size of the *Hsp70* gene is 1931 bp and a piece of DNA of approximately 2kb was amplified using the primers Pt-ex1 and Pt-ex2 see Fig 5.2. This DNA was sequenced from both internal and overlapping primers to give a complete sequence which was then compared with the sequence of the genomic DNA using the GAP programme from GCG; the results are shown in Appendix 4. The single intron from bp102-145 can be seen as a gap, apart from which, there is 100% concordance between the sequence derived from the cDNA and that derived from genomic DNA. No modification to the theoretical translated amino acid sequence of the protein is therefore required and this remains as in Fig 3.7.

5.2] Cloning of the *P. trichosuri* Hsp70 gene into an expression vector

The vector chosen for the expression of the coding sequence of the *P. trichosuri Hsp70* gene has been modified in our laboratory and is designated BQ1.1. Plasmid BQ1.1,

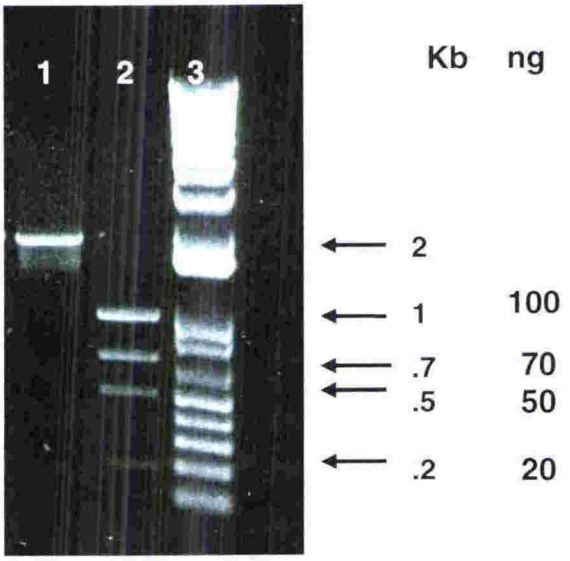


Fig 5.2 PCR amplification of coding sequence of the *Hsp70* gene from DNA extracted from *P. trichosuri* L3s using primers Pt-ex1 and Pt-ex2. The product of the expected size of 2kb is shown in lane 1, a mass ladder is shown in lane 2 and 1kb plus molecular weight markers in lane 3.

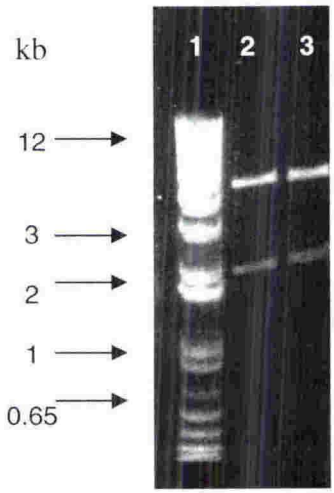


Fig 5.3 Analysis of plasmid DNA from cells transformed with pBQ1.1-PtHsp70.
The plasmid was digested with *Not1* and *Nde1* to show the presence of the cloned insert. Lanes 2 and 3 show the presence of an insert of the expected size, approximately 2Kb, corresponding to the coding sequence of *PtHsp70*

outlined in Fig 5.1, is an inducible arabinose promoter vector [Guzman *et al.*, 1995]; coding fusions are to a *NdeI* site which allows in frame initiation from the coding sequence's own start codon. The *NotI* site at the carboxyl terminus of the sequence allows fusion to a hexahistidine tag before termination, this facilitates purification of the expressed protein.

The purified cDNA from the PCR reaction product was digested by *NotI* and *NdeI* and ligated into vectorBQ1.1. The new plasmid was successfully transformed into XL2 cells as shown by the growth of many colonies compared with no colonies from cell transformed with vector DNA only. Restriction digestion of plasmid DNA prepared from cultures grown from six of these colonies showed the presence of inserts of 2.0kb, the expected size, in three of the six preparations, Fig 5.3, and suggested that these clones would express recombinant Hsp70.

5.3] Expression and purification of recombinant protein

Initial investigation of this expression was done by small-scale protein expression from six clones; results of SDS-PAGE from these inductions are shown in Fig 5.4. Comparing the induced and un-induced samples, high levels of expression of a protein of 70kD were seen in the insoluble fractions of clones BQ1.1-4 and –5. Protein was present in other samples but there was little observable difference in amount between induced samples and un-induced controls. The difference in the observed level of protein that was produced on induction is not unusual; it is possible that different clones may have grown from cells that had been transformed with different copy numbers of the plasmid but slight differences in environmental conditions can also cause differences.

Clone BQ1.1-4 was selected for the preparation of recombinant *P. trichosuri* Hsp70 protein and a 1L culture was grown and induced. The post-sonication supernatant was passed through a nickel IMAC column to trap any –His tagged protein present in the soluble fraction. Fig 5.5 shows substantial amounts of 70 kD protein in fractions eluted with 40-80 mM imidazole, however, there is a large amount remaining in the column flow-through due either to insufficient column capacity or low binding of the tagged

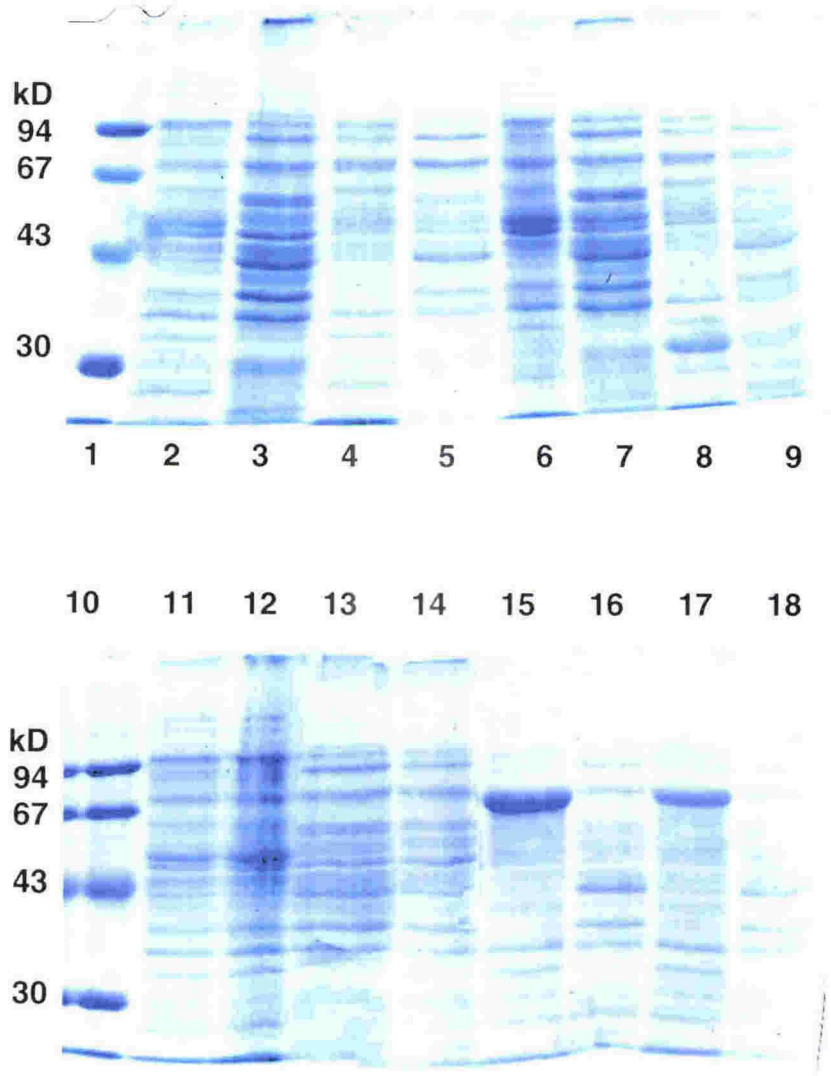


Fig 5.4 Small-scale protein expression from six clones containing the plasmid BQ1.1.

Single colonies were grown from transformation of XL-2 cells with plasmid BQ1-1 containing the coding sequence for PtHsp70. Colonies were induced with 0.2% arabinose for 4hr. Control cultures were not induced. 500µl of all cultures were pelleted and resuspended in 50µl B-Per reagent, centrifuged at 15,000g for 15min. Supernatnant was the soluble fraction; the pellet was resuspended and investigated as the insoluble fraction. Polacrylamide gel electrophoresis of 15µl of all samples was carried out on a 10% acrylamide minigel run at200 V for 2hr.

Fraction:

Lane 1: MWM
Lane 2: cl-1 induced, soluble
Lane 3: cl-1 induced, insoluble
Lane 4: cl-1 un-induced, soluble
Lane 5: cl-1 un-induced, insoluble
Lane 6: cl-2 induced, soluble
Lane 7: cl-2 induced, insoluble
Lane 8: cl-1 un-induced, soluble
Lane 9: cl-1 un-induced, insoluble
Lane10: MWM
Lane 11: cl-5 un-induced, insoluble
Lane 12: cl-4 un-induced, insoluble
Lane 13: cl-5 un-induced, soluble
Lane 14: cl-4 un-induced, soluble
Lane 15: cl-5 induced, insoluble
Lane 16: cl-5 induced, soluble
Lane 17: cl-4 induced, insoluble
Lane 18: cl-4 induced, soluble

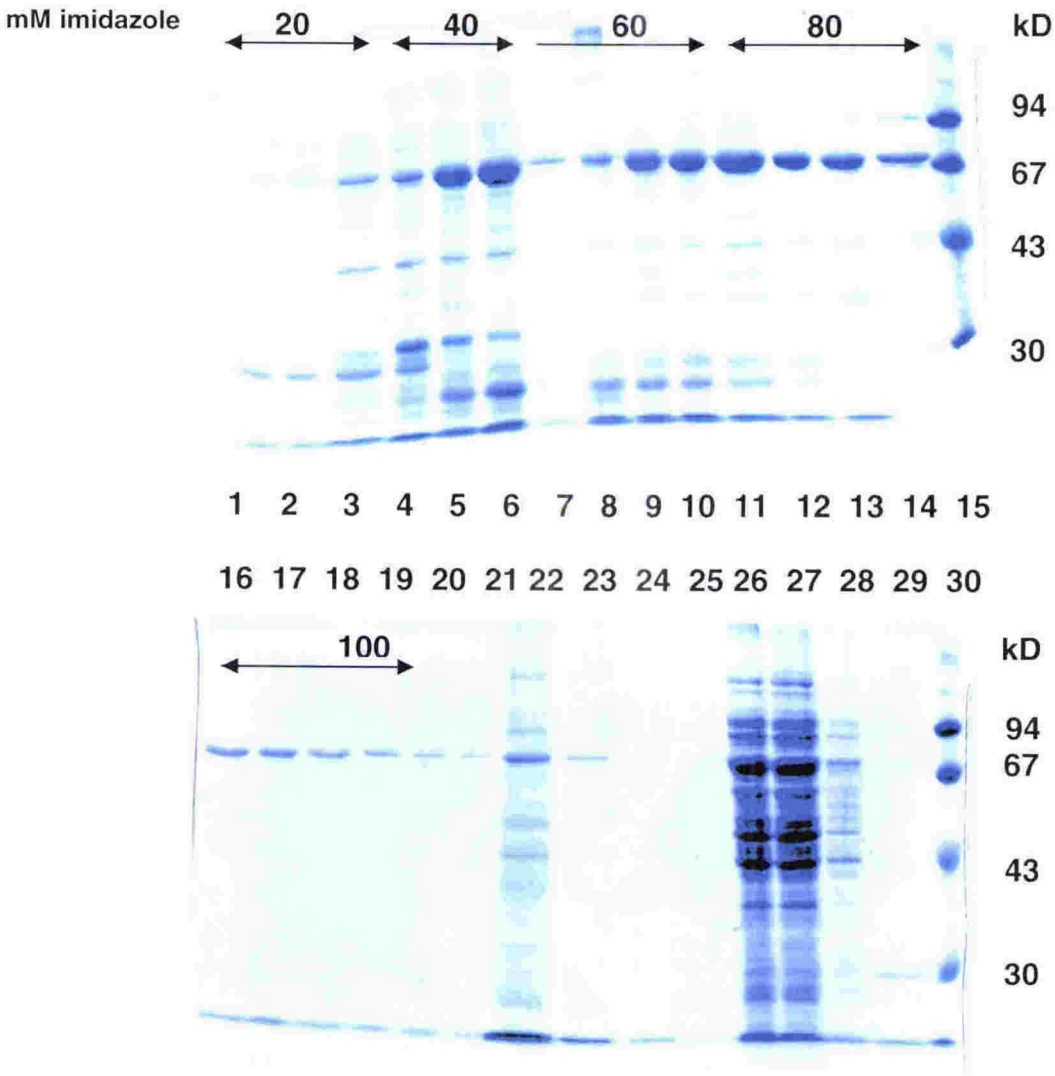


Fig 5.5 Isolation of recombinant –His tagged protein by Ni affinity chromatography.

A culture grown from clone BQ1.1.4 [see Fig 6.4] was induced with 0.2% arabinose, cells were pelleted and sonicated. Supernatant from the sonicated cells, the soluble protein fraction, was applied to a HiTrap chelating column. His-tagged protein was eluted with increasing concentrations of imidazole and electrophoresed on a 10% acrylamide minigel at 200V for 2hr

Electrophoresis fractions:
Lanes 1-19: fractions eluted from the column with increasing concentrations of imidazole, from 20 – 100mM.
Lane 20,21: fractions eluted from the column with EDTA pre stopping
Lane 22,23: fractions eluted from the column with EDTA post stopping
Lane 24,25: fractions from column clean-up with 0.5M $NaHPO_4$/5M NaCl
Lane 26: sonicated supernatant- pre chromatography
Lane 27: fraction from column flow-through- post binding
Lane 28,29: fraction from column wash- pre elution
Lane 30: MWM

protein. The partially purified protein from the nickel column was further purified by FPLC and results are shown in Fig 5.6. Although a large amount of protein has been lost in the purification process, reasonably clean samples with concentrations of 80μg/ml for the "dilute" recombinant protein and 170μg/ml for the "concentrated" sample from the first peak were obtained.

Considerable amounts of recombinant protein were present as inclusion bodies in the insoluble fraction after sonication so preparation of the protein from this fraction also was carried out; Fig 5.4, lanes 15 and 17 show the large amount of 70kD protein present in the insoluble fraction containing the inclusion bodies. This protein was isolated and purified with a high yield of reasonably pure protein being obtained from the cobalt affinity chromatography, shown in Fig 5.7. Further purification by FPLC was carried out with no increase in purity. When protein samples were run on a 15% gel, instead of the customary 10% gel, almost equal amounts of the expected 70kD protein and a low molecular weight protein of about 20kD were seen. This was surprising as the original protein had been concentrated through a 30kD cut off membrane and the lower molecular weight contaminant should therefore have been removed in the flow through. Nor was it removed by FPLC, see Fig 5.8; this indicates that it may be a breakdown of the 70kD protein. Consequently, although much lower in concentration than that purified from inclusion bodies, the 70kD soluble protein was used in further procedures.

5.4] Immunoblotting of the recombinant protein with possum antibodies

Recombinant protein, prepared from both the soluble fraction and the inclusion bodies, was immunoblotted with possum serum that contained antibodies to the original 70 kD antigenic protein of *P. trichosuri* larvae. After purification the soluble protein, along with a sample of insoluble non-purified protein and larval antigen were separated by electrophoresis, transferred to nitrocellulose and blotted as described. A sample of native larval antigen was also immunoblotted with the same possum sera. The results can be seen in Fig 5.9. With the native antigen three of the four positive sera had reacted with two proteins in the 67-75 kD region and the fourth serum had possibly reacted also, although the bands are blurred. These antibodies almost certainly correspond to those antibodies to proteins in the 70 kD region seen in the serum of many infected possums, Fig 5.9 can be compared with Fig 2.5. Non-infected, negative,

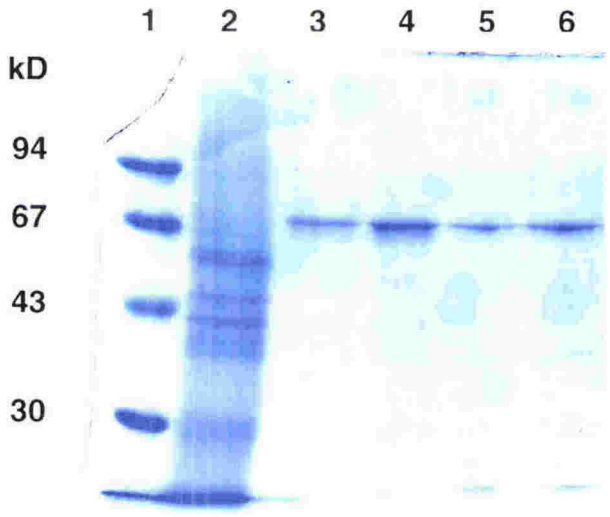


Fig 5.6 Soluble recombinant protein, isolated by Ni affinity chromatography was further purified by FPLC on a Superdex™ 75 separating column. Protein samples from the first two peaks were collected and concentrated either by centrifugation through a 30 kD membrane at 5000g for 30min- denoted "dilute" protein- or centrifuged at 600g for 90min denoted "conc" protein. The fraction were electrophoresed on a 10% acrylamide minigel at 100V for 2hr.

Lane 1: MWM
Lane 2: Native Pt L3 antigen. Volume loaded = 20µl
Lane 3,: "dilute" protein from FPLC peak 1. Amount loaded =1.6µg
Lane 4: "conc" protein from FPLC peak 1. Amount loaded =3.4µg
Lane 5: "dilute" protein from FPLC peak 2. Volume loaded = 20µl
Lane 6: "conc" protein from FPLC peak 2. Volume loaded = 20µl

Protein present in peak 2 appears to have the same MW as in peak 1.

Fig 5.7 Purification of recombinant 70kD protein from inclusion bodies.

A culture derived from clone BQ1.1.4 was induced with 0.2% arabinose; cells were pelleted and sonicated. Protein present as inclusion bodies was solubilized in 8M urea buffer and centrifuged at 15,000g for 30min. His-tagged protein was separated by affinity chromatography on a TALON™ cobalt based column and eluted with increasing concentrations of imidazole. Fractions were electrophoresed on a 10% acrylamide minigel at 200 V for 2hr.
Fractions eluted with 20mM imidazole are shown prior to purification by FPLC.

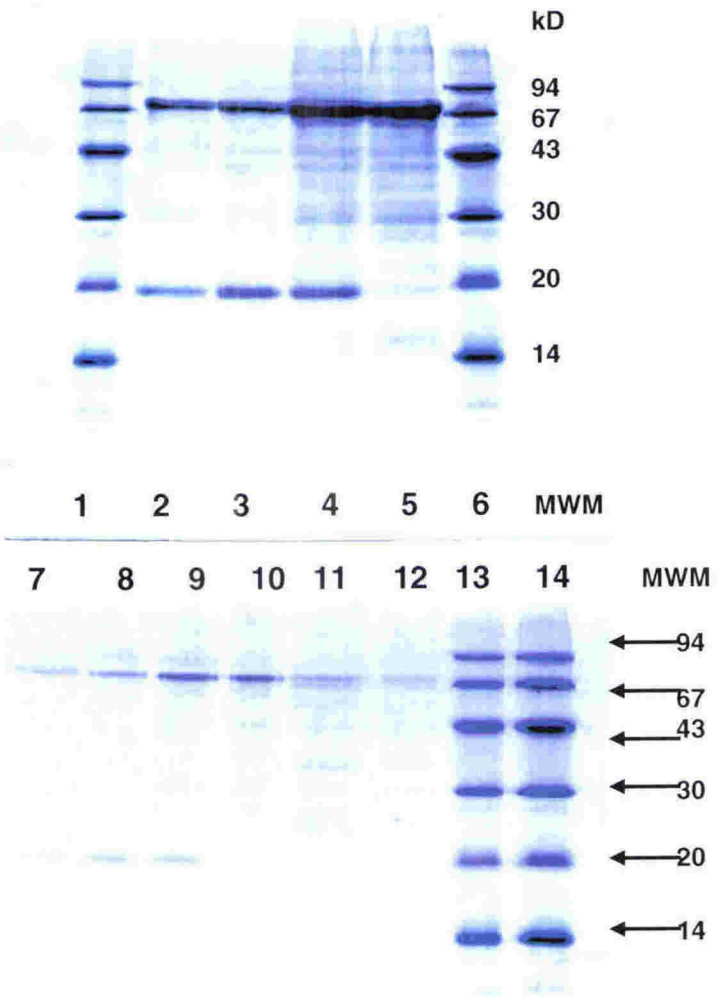


Fig 5.8 Presence of contamination in PtHsp70 protein purified by FPLC.

Recombinant PtHsp70 protein samples derived from solubilized inclusion bodies were purified by metal-affinity chromatography and FPLC and were concentrated by centrifugation through a 30kD membrane. Protein samples were electrophoresed on a 10% acrylamide minigel at200 V for 2hr.

70kD protein and also 20kD contaminating protein can be seen in lane 2 [sample 1, amount loaded = 3μg], lane 3 [sample 2, amount loaded = 5μg], lane 4 [sample 3, amount loaded = 14μg], and lane 5 [sample 4, amount loaded = 10μg]. Lanes 7,8,9,10 contain a 1:10 dilution of samples 1-4.
The contaminating 20kD protein is not present in samples of soluble recombinant protein [lane 11, amount loaded = 0.8μg, lane 12, amount loaded = 1.7μg. Molecular weight markers are shown in lanes 6, 13,14.

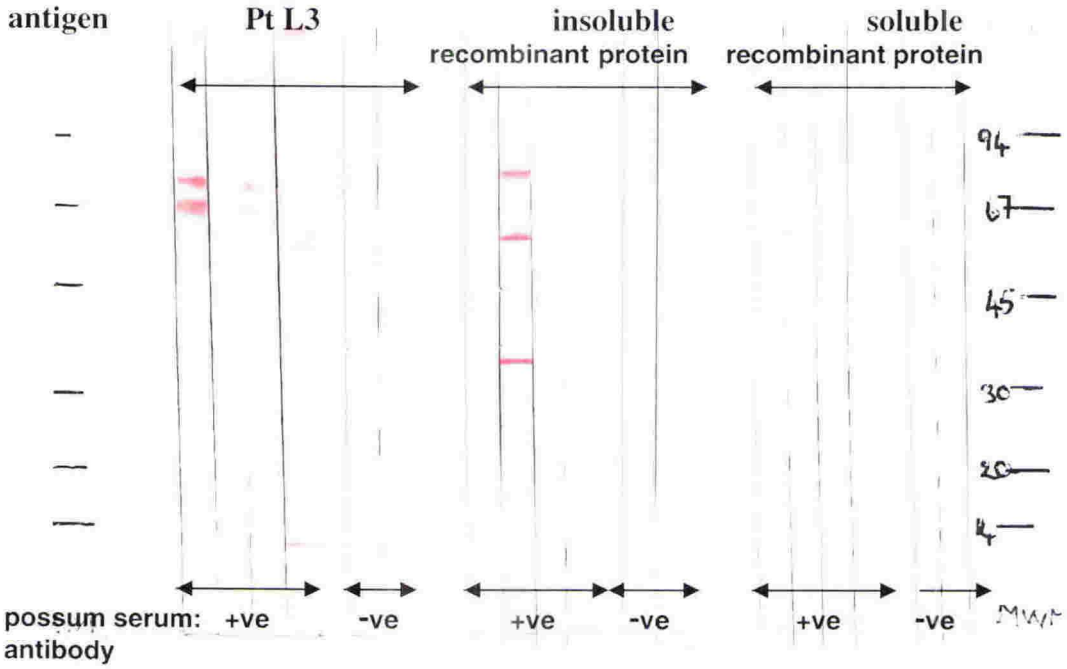


Fig 5-9 Immunoblott of recombinant PtHsp70 protein compared with native *P. trichosuri* L3 antigen.

P. trichosuri L3 larval extract, purified soluble and partially-purified insoluble recombinant protein were separated by electrophoresis on a 5-25% gradient acrylamide gel and transferred to nitrocellulose. Nitrocellulose strips from each of the three samples were blotted with possum serum from four different possums that had shown the presence of antibodies to 70kD proteins on previous blots – positive serum [lanes 1-4, 7-10, 13-16]. Serum from Stewart Island possums that have never shown the presence of these antibodies was used as negative control serum [lanes 5-6, 11-12, 17-18]. All serum was used at a concentration of 1:1000.

possum sera did not react. There were a few very faint bands seen in the 67-75kD range with the insoluble Hsp70 strips but no reaction at all with the soluble protein. It had been expected that recombinant protein would give a single clear well defined band when probed with serum that had been shown to contain antibodies but from Fig 5.9 it can be seen that the antibodies did not react with recombinant protein in a similar way to the native protein. Therefore, the recombinant protein is not immunologically identical to the native protein.

5.5] Comparison of recombinant and native 70kD protein

The recombinant and native 70kD protein had reacted differently with possum serum antibodies and so it could not be confirmed that the recombinant protein, translated from the cDNA of a Pt *Hsp70* gene, was identical to the protein that had been initially investigated. Therefore, the native and the recombinant protein were compared further by mass fingerprinting of peptides derived from tryptic digestion using MALDI-TOF mass spectrometry. An immobilized gradient IEF system followed by second dimension SDS-PAGE allowed quick preparation of a sample of the native 70kD protein in quantities that were sufficient for the MALDI-MS but would have been insufficient for protein identification by Edman degradation; the IPGphor™ 2D system was not available to us when the protein was first being isolated.

A sample of larval antigen with a high intensity band at 70kD, as seen on a traditional 1D SDS PAGE gel, was used for the 2D gel. The pI of the Hsp70 protein had been determined as being approximately between pH5-6 and so a narrow pH range immobilized pH gradient of 4-7 was selected to obtain the highest possible resolution of the mix of proteins. Two-dimensional electrophoresis gave reasonably good separation of the proteins; unfortunately no sample that had not been separated by IEF was run on this gel for comparison but a 1D gel of the antigen used can be seen in Fig 2.11. As can be seen in Fig 5.10, there are a number of individual spots between 67 and 80 kD, and six or seven of these appear to have an almost identical molecular weight. The immunoblot from the duplicate gel can be seen in Fig 5.11; some non-specific staining can be seen corresponding to the protein smears at the sides of the dry strip but very little else can now be seen on this blot. At the time of development, however, two or

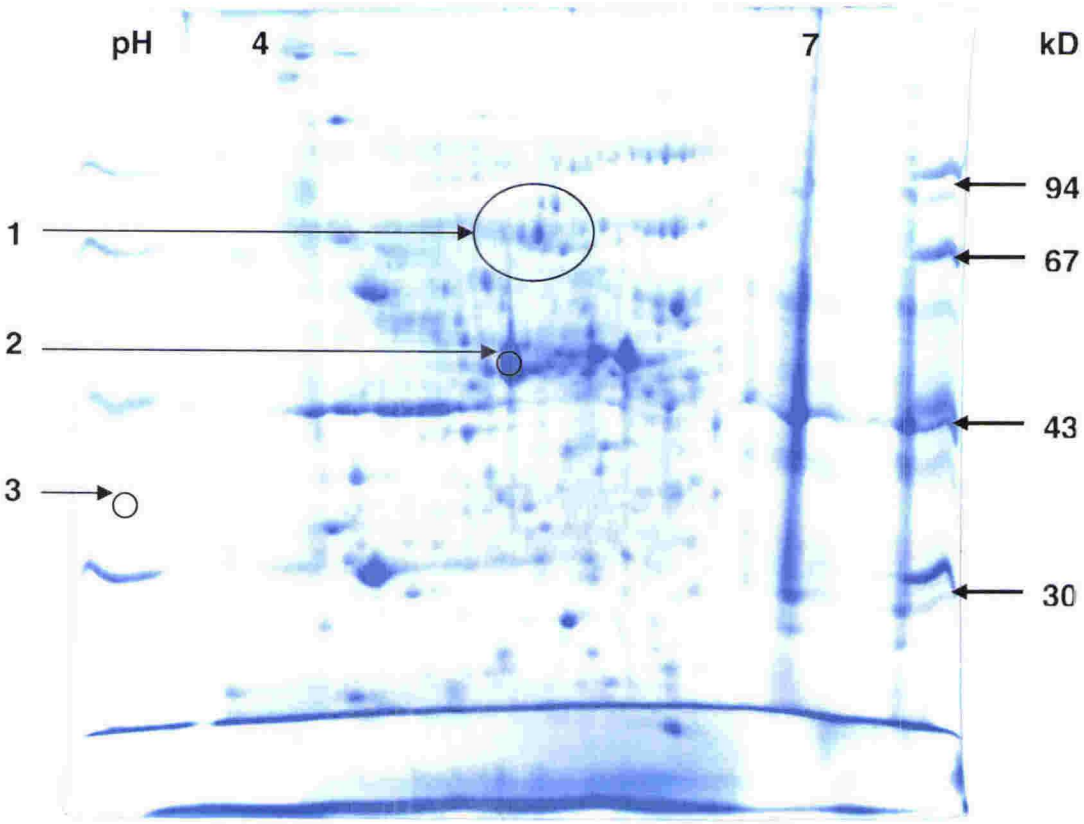


Fig 5-10 Two dimensional electrophoresis of native *P. trichosuri* L3 antigen

P. trichosuri L3 proteins [300µl of standard PtL3 antigen] were separated by isoelectric focussing on an Immobiline Dry Strip, pH4-7, for 52,000 Total Volt hours. After standard equilibration, 2nd dimension electrophoresis was carried out on a 10% SDS-PAGE gel, at 150V for approx. 10hr

◯ shows the sample areas where protein spots were excised for peptide mass analysis.

1: spots labelled 70-1 to -5, see enlargement, Fig 5.12.
2: actin
3: blank piece of gel use for negative control.

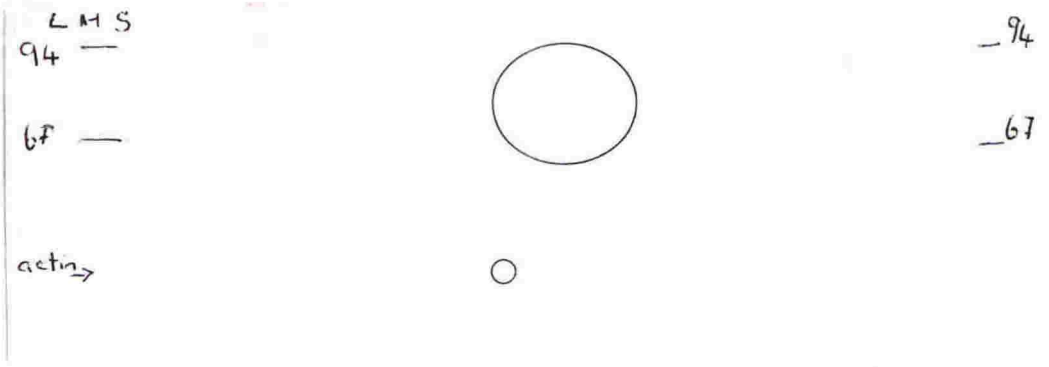


Fig 5.11 Immunoblot of two dimensional gel of native *P. trichosuri* L3 proteins.

P. trichosuri L3 proteins [300µl of standard PtL3 antigen] were separated by two dimensional gel electrophoresis [a duplicate of the gel in Fig 5.10]. The gel was transferred to nitrocellulose and the area of the 2D Western blot from approximately 43-94kD was blotted with possum serum positive for antibodies to 70kD proteins on previous blots, serum at 1:500.

Circled area corresponds to circled area in Fig 5.10.

three faintly staining spots could be seen around the location of dots seen also on the gel. These positive spots were circled on the blot then five spots from this area were excised as shown on the close up, Fig 5.12. All these proteins, 70-1 to 70-5, were considered to have reacted with the antibodies, as the immuno-stained dots were too diffuse to distinguish between them. A few other proteins of different molecular weight also reacted with the probing possum serum; most of these have also faded and can no longer be seen on the nitrocellulose.

All protein samples were digested with trypsin, and the mass of the resultant peptides measured by MALDI-TOF mass spectrometry and compared with database masses. All samples were clean and gave signals of reasonable strength. The results of the ProFound analysis can be seen in Table 5.1.

Trypsin theoretically cleaves *P. trichosuri* Hsp70 into 82 peptides; in all cases the number of peptides whose masses were determined, was considerably fewer, at around 47. Also, in all cases, there were at least as many unmatched as matched peptides. Despite this, there is a 99% probability that spot 70-3 from the native protein mix is the same protein as that theoretically translated from the genomic sequence for *P. trichosuri Hsp70*, and the recombinant protein, made from the coding sequence for this gene, has been identified as a *P. trichosuri Hsp70* with the same high probability. Spot 70-1 has also been identified as a *P. trichosuri* equivalent of a human ATPase, H^+ transporting lysosomal [vacuolar proton pump] alpha polypeptide, and there is a tentative identification for 70-4 as a Diacyl glycerol kinase. The spot that is known to be Actin, and is used as the positive control, was identified as such but less conclusively than expected.

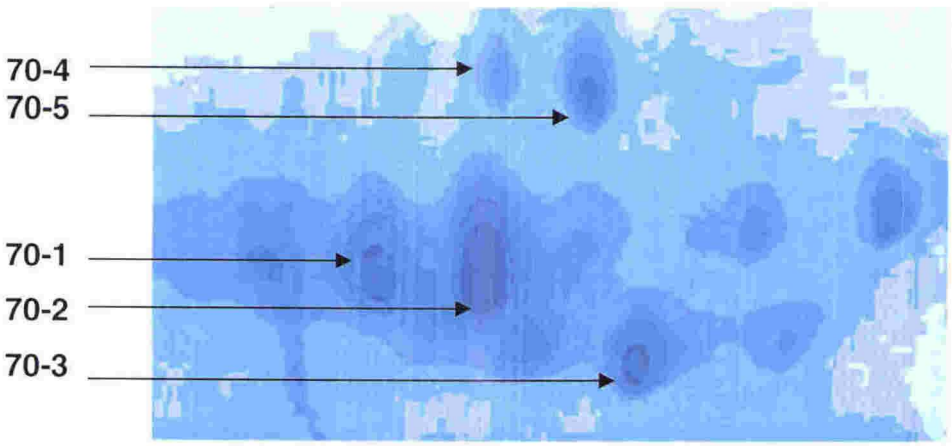


Fig 5.12 Enlargement of the circled area from the 2D gel shown in Fig 5.10 and the Immunoblot shown in 5.11.

Individual spots that were of approximately 70kD in size and pI 5-6, and that had shown some positive reaction with antibodies in positive possum serum, were excised for peptide mass analysis.

The position of the excised spots labelled Pt70-1, -2, -3,-4 and -5 is shown

Table 5.1 Identification of proteins by mass fingerprinting using ProFound

Sample	Probability	Est'd Z	Coverage	Protein Identity
70-1	1.0	1.87	20	ATPase vacuolar protein pump (*H. sapiens*)
70-2				no significant matches with any protein in database.
70-3	1.0	2.36	34	Hsp70 *P. trichosuri*
70-4	1.0	1.47	22	Diacyl glycerol kinase - several species
	9.9e-001	0.98	17	elongation factor 2
70-5				no significant matches with any protein in database
Actin	$9.9e^{-001}$	0.89	30	Actin 4 (*C. elegans*)
Rec 5	1.0	2.16	22	Hsp70 (*P. trichosuri*)
rec 0.5	9.8^{-001}	0.81	19	Keratin - no significant match. Highest ranked match is to the contaminant human keratin

Samples: 70.1 to 70.5 are shown on Fig 5.12, Actin is shown in Fig 5.10, Rec 5 and rec 0.5 are samples of recombinant protein.

Probability: Profound computes normalized probability that a protein in the database is the protein being analyzed. Currently a probability score of 1 is usually required to make an identification by Profound.

Z Score: is an indicator of the quality of the search result. How significantly an identified candidate is different from random matches with the database.

Z Score	Probability
1.282	0.900
1.645	0.950
2.326	0.990
3.090	0.999

Coverage: is defined as the ratio of the portion of the protein sequence covered by matched peptides to the whole length of protein sequence. Generally a good match will have a minimum coverage of 20%.

Protein identity: The peptide masses were searched by ProFound (Proteometrics, version 4.10.5) against the NCBI database. The highest ranked match is shown unless P is less than 9.9e-001.

Discussion:

A recombinant protein was successfully made from the coding sequence for an *Hsp70* gene from *P. trichosuri*. When this was compared with the native protein using the original detection system –infected possum serum- the two proteins reacted differently. This difference in response was investigated by mass fingerprinting which confirmed that one of the original 70kD antigenic proteins was a *P. trichosuri* Hsp70 and that the purified recombinant protein also corresponds to a *P. trichosuri* Hsp70. Both are more like the theoretical protein translated from the genomic sequence for *P. trichosuri Hsp70* entered into the database than Hsp70s from any other source.

The mass fingerprinting method is considered to be a powerful way of comparing an unknown protein to all others in the databases used and of obtaining a possible identity, but in this case it is not a sensitive way of discriminating between proteins of very similar mass and sequence. It is inferred that the difference is not a slight variation in secondary structure as both recombinant and native protein were run on a denaturing gel prior to immunoblotting. Both proteins may well be members of the *P. trichosuri* heat shock 70 family of proteins yet differences do exist.

In all organisms investigated so far there are several *Hsp70* genes coding for heat inducible or constitutively expressed proteins involved in a wide range of functions in various sub-cellular compartments [Feige and Polla, 1994, Mayer and Burkau, 1998]. It would be expected that *P. trichosuri* would have several *Hsp70* genes; recently three expressed sequence tags [ESTs] with similarity to *Hsp70* genes, derived from a *P. trichosuri* expression library have been added to the GenBank database [GenBank accession no. BI703733, BI742938, BI743201]. Two of these appear to be from the same gene and one from a second distinct gene. Heat shock 70 proteins all show a high degree of similarity with possibly many similar trypsin cleavage sites but they are functionally distinct and probably immunologically distinct as well. As discussed in Chapter III, it is a feature of Hsp70s that they are both extremely conserved and at the same time are exquisitely specific in their antigenicity [Moseley, 2000]. The gene that has been expressed may not be the *Hsp70* to which the possum antibodies were made despite sharing much of the highly conserved sequence.

Considering the antigenicity of closely related Hsps it is interesting to note that in mammalian cells an Hsp70 molecule with a specific antigenic peptide bound in its cleft elicits an antigenic response whereas a purified protein with no peptide does not [Suto and Srivastava, 1995, Blachere *et al.*, 1997]. This may be a general feature; it is likely that a recombinant protein would not be associated with the same peptide as a native protein as peptides from the nematode larval environment would not be available to the recombinant protein. Whether such peptides would remain associated with their Hsp molecule under denaturing conditions, as occur in SDS-PAGE prior to immunoblotting, is questionable.

Proteins where the natural availability is limited are generally obtained by gene cloning and expression in an *E. coli* system. This is usually the system of first choice as it is well established, relatively simple and has proven successful for numerous proteins from many sources [Marston, 1986]. Both nematode proteins and Hsp70 proteins and nematode Hsp70 proteins eg that of *Trichinella britovi* have been made in such a system [Vayssier *et al.*, 1999].

There are, however, disadvantages in expressing eukayotic genes in a prokaryotic system. Often they may be expressed at high levels where the hydrophobic interaction between proteins may prevent correct folding and the formation of the native cysteine disulphide bonds. Consequently they aggregate in insoluble inclusion bodies and purification requires solubilization in denaturant. Reconstitution to a native configuration is required before an active form of the protein results [Fischer *et al.*, 1992, Lilie, 1998]. Other post-translational modifications such as glycosylation are also lacking or abnormal in protein expressed in such a system.

It is possible to redress some of these inaccuracies in expression by using a eukayotic expression system such as yeast, insect or mammalian cell lines. Transgenic *C. elegans* itself has been used as an expression system by the overexpression of a nematode gene product in a microinjected worm [Kwa *et al.*, 1995, Redmond *et al.*, 2001]. The advantages of using *C. elegans* for nematode proteins include its closeness in evolutionary terms, its ability to transplice proteins in a similar way to other nematodes

and the existence of a modified worm would allow functional studied at the same time. This is an interesting variant on the transgenic animal theme.

To summarize: the recombinant protein has been translated from the coding sequence of a *P. trichosuri Hsp70* gene and has been identified as such by mass fingerprinting. The recombinant protein does not react with possum serum. A group of proteins with very similar molecular weights was isolated from native larval antigen. One of these is a *P. trichosuri* Hsp70 protein as identified by mass fingerprinting. It was considered that all members of the group that were excised for mass fingerprinting did react with possum serum although protein 70-3 reacted weakly, certainly less strongly than a similarly sized protein [70-2] on the immunoblott, Fig 5.11. Little sign of that reaction appears to have remained and this must throw doubt on whether 70-3, PtHsp70 protein is in fact antigenic.

These results can be compared with those of the preparative electrophoresis experiment in Chapter II.4; here, there were samples where there was one strong band of protein and several weak bands on the gel. The predominant protein in one such sample [Fraction 8, Fig 2.14] was sequenced and shown to have homology with the N-terminal signature motif of the Hsp70 proteins. At least two proteins of close molecular weight were separated by this method and at least two of these proteins reacted with antibodies from possum serum. It would seem likely that the antibodies were reacting with the predominant protein but in Fig 2.14, lane 6 there is no visible protein band seen with silver staining on the gel whereas there is a visible reaction with possum antibodies, seen in Fig 2.15. It is therefore possible that a similarly sized second protein was present in the sequenced sample at a far lower concentration and it was this protein that reacted with the serum antibodies; the identity of this possible unseen protein is unknown. Hsp70 proteins are highly immunogenic but there was a lack of response of recombinant Hsp70 from the *P. trichosuri* gene. The identity of the original target antigenic protein must remain in doubt.

Chapter VI Conclusions

Starting from the initial observation of an antibody response in some possums, it was confirmed that a set of immunogenic proteins was present in *P. trichosuri* that could be detected by antibodies from the serum of possums infected with this parasite but not with others. A short amino acid sequence was obtained from the immunogenic protein; this was sufficient to identify it as a heat-shock70 protein and not a secreted protein, which was contrary to expectations. A gene and upstream sequences for one heat shock protein were segregated from a *P. trichosuri* genomic library and a putative promoter cloned into a vector upstream of a *lacZ: gfp* reporter construct. The decision to use parasitic adults was taken and the extraction and culture of these adults carried out. This sequence gave rise to reporter gene expression in the offspring of injected parasitic worms. The cDNA for the protein was amplified, cloned into an expression vector and the recombinant protein expressed in *E. coli*; this was compared with the native Hsp70 protein. Conclusions drawn from results of the successive parts of this project have been discussed in their various chapters.

Perhaps the most important result from this work was showing that the creation of a transgenic *P. trichosuri* was feasible; the following section puts this achievement into context. Transgenic animal technology in *C. elegans*, coupled with genomic and bioinformatic techniques, has become a powerful tool for examining the spatial and temporal expression patterns of genes, investigating gene regulation, and determining the function of gene and gene products in an organism. This latter can be done by either a gain of function analysis (by ectopic or over expression of the gene and mutant rescue) or loss of function analysis by gene knock-out using the Tc1 transposon or chemical mutagenesis, or silencing by double stranded or antisense RNA [Bürglin *et al.*, 1998].

The use of *C. elegans* as a heterologous system for studying the regulation and expression of genes from parasitic nematodes of both medical and veterinary importance has been advocated as there is no parasitic system in which this can be done at present [Grant, 1992, Hashmi *et al.*, 2001]. The expression in *C. elegans* of a *lacZ*

reporter gene under the control of promoters from a cysteine protease gene and a pepsinogen gene from *H. contortus*, a cuticular collagen gene from *Ostertagia circumcinta* [Britton *et al.*, 1999], and a GAPDH gene from *Globodera rostochiensis* [Qin *et al.*, 1998] shows conservation of regulatory sequences and transcription machinery between the nematode species. The expression in *C. elegans* of *H. contortus* pepsinogen under the control of a *C. elegans* promoter [Redmond *et al.*, 2001], the expression of β tubulin genes from *H. contortus* in a biologically active form [Kwa *et al.*, 1995], and the expression of extracellular glutathione S-transferase from *Onchocerca volvulus* [Krause *et al.*, 2001] confirms that *C. elegans* can be used as a model to obtain information about gene function in a parasitic species and may even be used as a system for expressing parasite proteins.

Despite this, there are obvious differences between a free-living soil organism and an organism that has adapted to parasitic life. The usefulness of the model may depend on how closely its biology corresponds to that of the parasite e.g. how closely is dauer formation in *C. elegans* equivalent to development of infective larvae in parasitic organisms; will the genes involved in the former equate with those of the latter. It would seem, therefore, to be of value to extend the technique of transgenesis to other nematodes, those that are parasitic to plants or animals and are of medical and veterinary importance. However, achieving stable transgenic lines of nematodes has proved difficult other than for the free-living animals of the *Caenorhabditis* and *Panagrellus* genera.

One organism in which this has been achieved is *Heterorhabditis bacteriophora,* a small soil dwelling nematode in which infective third stage larvae enter soil insects and release symbiotic bacteria [*Photorhabdus luminescens*] from their gut which reproduce, killing the insect host and providing a suitable food source for the multiplying nematodes. This organism and other species of *Heterorhabditis* and *Steinernema* are useful biocontrol agents against a number of insect pests but their sensitivity to stress, especially increasing temperature and desiccation reduces their usefulness. *H. bacteriophora* has been transfected by microinjection and transgenic offspring expressing the *lacZ* reporter and the *rol6* gene under the control of the *C. elegans hsp16* promoter have resulted [Hashmi *et al.*, 1995a]. Transformation of the same organism

with the same construct was also successfully carried out using a microprobe array [Hashmi *et al.*, 1995b].

Subsequently, five to ten copies of the *C. elegans hsp70*-A gene were introduced into *H. bacteriophora* with greatly increased survival after lethal temperature exposure for the transgenic nematode [Hashmi *et al.*, 1998]. These nematodes, genetically enhanced for increased thermotolerance, have been released into the environment where they developed normally and persisted as well as the wild type strain [Gaugler *et al.*, 1997]. Transfection caused no change in virulence to their insect hosts [Wilson *et al.*, 1999]. This is the first example of a genetically engineered organism being released into the environment and used for biocontrol.

Attempts to increase the resistance to drought of another genus of entomopathogenic nematode have been made. In these, *Steinernema feltiae* were transfected with the *tps-1* gene from yeast coding for trehalose-6-phosphate synthase under the control of the *C. elegans hsp*16 promoter. Transgenic progeny were found with a greatly increased tolerance to dehydration and this correlated with the presence of the transgene as certified by single-animal PCR but the transformation was not stable [Vellai *et al.*, 1999]. Transgenic *Heterodera glycines* have been made by injecting males then crossing them with the females; the construct consisted of the *C elegans myo 3* promoter and *gfp* as the reporter and high levels of expression were seen [Opperman and Bird, 1998].

Transfection of *Litomosoides sigmodontis* by ballistic DNA transfer of *gfp* under the control of a *C. elegans* actin-1 promoter gave rise to expression in the injected adults both *in vitro* and after re-implantation in their host. Transgene expression was also seen in a few microfilaria [Jackstadt *et al.*, 1999]. No stable heritable transformation has been reported to date. Ballistic techniques were also used to introduce a marked spliced leader [SL] RNA gene under control of the *Ascaris* SL-RNA promoter into *Ascaris suum* embryos. These embryos with the inserted gold plus DNA particles continued to develop during the 80 hours of the experiment and marked SL-RNA was expressed. A luciferase gene, again driven by the *Ascaris* SL-RNA promoter was also expressed for the duration of the experiment and a reasonably high percentage of transgenic embryos resulted. This method was aimed at transient expression only [Davis *et al.*, 1999].

Strongyloides stercoralis is a significant intestinal parasite of humans and other *Strongyloides* species infect other mammals. These parasites have a life cycle similar to *P. trichosuri* in that they have both a free-living and parasitic reproductive phase. Recently, microinjection of free-living females with a *gfp* reporter construct under the control of an autologous *actin-2* promoter and *era-1* promoter [for an endoplasmic reticulum ATPase] gave rise to expression in female parental tissues and in some embryos. None of the transfected embryos hatched [Lok and Massey, 2002].

In the experimental work with *P. trichosuri* the introduced reporter gene under control of the Pt *Hsp70* promoter was transfected into the parasitic adult and was expressed in the free-living adult in the alternative development pathway after several generations. It is the only heritable transformation of a vertebrate endoparasite, an important group both medically and economically, achieved to date.

Several nematodes have been suggested for the role of model parasite and, with the advent of transgenesis, *P. trichosuri* looks like a good candidate. There are advantages in using a parasitic organism that has a life cycle phase with accessible and manipulable reproductive adults. The organism can be maintained *in vitro* almost indefinitely and the number of progeny with a transformed phenotype can be amplified by successive free-living cycles before any recourse to the host is necessary.

An expressed sequence tag [EST] programme is underway for this parasite and nearly eight thousand ESTs have been sequenced [as of 2/02] from a *P. trichosuri* expression library and are available in the database. This will provide an excellent platform for future functional genomic projects.

New molecular phylogenies for the Nematoda have suggested that parasitism may have arisen independently on several occasions [Blaxter *et al.*, 1998]. These have defined the relationship of *C. elegans* to the main parasitic groups; it is most closely related to *Heterorhabditis* and the vertebrate parasites of the Strongylida. *P. trichosuri* at present classified as Rhabdita, Strongyloididae, could be a useful model for a particular sub-set of the parasitic members of the phylum, perhaps the mammalian parasites of the genus *Strongyloides*. The sequence obtained in this project, which is still the only genomic

sequence for this organism to date, could be used for initial molecular phylogenetic studies, as despite the drawbacks already mentioned in Chapter III, *Hsp70* molecules are considered useful and legitimate molecules for evaluating phylogenetic relationships because they are so highly conserved and have homology across the entire length of the molecule giving rise to many informative characters [Ruef *et al.*, 2000].

The value of *P. trichosuri* as a model parasite and the ways it could be used is discussed in Grant and Viney [2001].

Future work: Development of a transgenic *P. trichosuri*.

There is a considerable amount of work needed to turn an occasional green worm into an efficacious possum killing or sterilizing agent. Firstly, new constructs for microinjection must be developed to bring about higher levels of transgene expression. The existing *Hsp70* promoter has been shown to have some function so attempts to enhance its activity, as discussed in Chapter IV, would be a starting point. New promoters from *P. trichosuri* orthologues of strong housekeeping or secretory genes from *C. elegans* should be sought and expression using non-specific promoters needs to continue also. Once enhanced expression has been achieved vectors comprising the control gene with appropriate regulatory sequences must be constructed. Ways of assaying for immediate and short-term activity of the control gene *in vitro* and *in vivo* must be developed; long term, the test for effectiveness is fewer possums!

There are several technical transfection methods that could be further pursued, ballistic, or biolistic, introduction could be tried and a return to attempts to microinject DNA into free-living adults.

It would be desirable to re-introduce injected adult parasites into the possum small intestine and monitor their establishment and egg production *in vivo*. This may allow the adults to produce greater numbers of transgenic offspring, also. And it will be necessary to induce transgenic offspring *in vitro* to enter the parasitic pathway and ascertain that they can infect the possum host and establish in the gut in the normal manner.

The whole gamut of epidemiological and ecological studies will need to be carried out with the new organism. Discussion of the extent and dynamics of population decrease is outside the scope of this project.

When and if a transgenic *P. trichosuri* is made that acts effectively in laboratory and contained field trials, there is yet another hurdle to be overcome. Will its use be permitted? Considerably more is known about the public's acceptance of genetically modified organisms now than when this project was conceived in the mid nineties. Then there was generally less adverse comment but also no data on acceptability. Since the start of this project the public's attitude to possums and possum control has been investigated [Parliamentary Commissioner for the Environment, 2000]. Compared to existing technologies, biocontrol is seen as more acceptable than widespread 1080 poisoning but less acceptable than manual methods, i.e. shooting and trapping. But as most biocontrol research has a component of genetic modification, discussion took place in the context of the public attitude to genetic engineering in general.

The Royal Commission on Genetically Modification has established that, generally, public opinion accepts genetic modification for medical or for some desired public good, while rejecting most purely commercial applications; conservation purposes are somewhere between these two positions [Report, Chapter 6, p107]. While there is no clear understanding by the general public of what this biocontrol entails or the possible future risks involved, the application of genetic engineering to possum control appears to fall between medical applications and the genetic engineering of food in terms of its acceptability. Specifically, for this project, delivery of biocontrol via a genetically modified parasite "was not considered unacceptable" [Parliamentary Commissioner for the Environment, 2000, p84]. Expert opinion continues to see the creation of a self-disseminating vector as an undisputed requirement of any control programme [Davies *et al.*, 2001].

This project, along with the work of others, has played a significant part in the creation of a transgenic *P. trichosuri*. The contribution to and place in the overall possum biocontrol project is as shown in Fig 1.9 at the beginning of this work. It is a small but necessary part of the development of a self-sustaining and disseminating vector that could drastically reduce the possum population of New Zealand.

Appendix 1:

Potential pathogens of brushtail possums

From: Report on the Survey of Potential Pathogens in Australian Brushtail Possums coordinated by Vernox Pest Management, Obendorf, D.

Agents causing morbidity and/or mortality in free-range possums:

Tuberculosis- *Mycobacterium bovis*

Agents associated with immune compromisation:

Toxoplasmosis- *Toxoplasma gondii*
Coccidiosis- *Eimeria sp*
Intestinal protozoan infections- ?*Entamoeba, Giardia, Crytpsporidia spp*
Bacterial infections- *Salmonella, Yersinia, Staphylococcus, Streptococcus spp*
Fungal infections- Adiaspiromycosis (*Emmonsia crescens*)
Candidiasis (*Candida albicans*)
Cryptococcosis (*Cryptococcus spp*)

Agents of significance as zoonoses:

Leptospirosis- *Leptospira serovar balcanica*
Ringworm- *Trichophyton mentagrophytes*
Trichinosis- *Trichinella pseudospiralis*
Q fever- *Coxiella burnetii*
Queensland tick typhus- *Rickettsia australis*
Arboviruses- *Alphavirus* (Ross river virus and Whataroa virus)
Flavivirus (Murray valley encephalitis virus)

Possible pathogens or vectors:

Nosema- *Eperythrozoon spp*
Herpesviridae- Marsupial herpes virus/ Parma wallaby herpes virus
Chlamydia psittaci- reproductive tract disease
Cryptococcosis- *Cryptococcus neoformans* var. *gatti*
Tyzzer's disease- *Bacillus piliformis*
? *Durikainema sp*
? *Marsupostrongylus spp*

Appendix 1:

Endoparasites of *Trichosurus vulpecula* and locations in which they were recorded from Viggers and Spratt 1995.

Parasite species	Australia						NZ
	Vic	NSW	SA	WA	Tas.	Qld	
Protozoa							
Eimeriidae							
Eimeria spp.	+				+		+
Toxoplasma gondii Nicolle and Manceaux 1909	+					+	
Sarcocystidae							
Sarcocystis spp.	+						
Endamoebidae							
Entamoeba sp.						+	
Cestoda							
Anoplocephalidae							
Bertiella trichosuri Khalil 1970	+	+	+		+		+
Trematoda							
Fasciolidae							
Fasciola hepatica Linnaeus 1758	+	+			+		+
Nematoda							
Rhabditoidea							
Strongyloididae							
Parastrongyloides trichosuri Mackerras 1959		+				+	+
Strongyloides sp.		+					
Trichostrongyloidea							
Herpetostongylidae							
Paraustrostrongylus trichosuri Mawson 1973	+	+	+			+	+
Trichostrongylidae							
Trichostrongylus colubriformis (Giles 1892)		+	+				+
Trichostrongylus axei (Cobbold 1879)			+				
Trichostrongylus rugatus Monnig 1925		+					
Trichostrongylus retortaeformis (Zeder 1800)							+
Trichostrongylus vitrinus Looss 1905		+					
Trichostrongylus sp.	+	+	+				+
Cooperia curticei (Giles 1892)		+					
Molineidae							
Nematodirus sp.		+					
Dromaeostrongylidae		+					
Filarinema trichosuri (Johnston and Mawson 1939)							
Peramelistrongylus sp.	+						
Profilarinema hemsleyi Durette-Desset and Beveridge 1981			+				
Profilarinema sp.		+			+		

Parasite species	Australia						NZ
	Vic	NSW	SA	WA	Tas.	Qld	
Metastrongyloidea							
Angiostrongylidae							
Marsupostrongylus minesi Spratt 1979		+					
Marsupostrongylus pseudominesi Spratt 1984						+[A]	
Marsupostrongylus longilarvatus Spratt 1979		+					
Filostrongylus tridendriticus Spratt 1984					+		
Oxyuroidea							
Oxyuridae							
Adelonema trichosuri (Johnston and Mawson 1938)	+	+	+			+	
Ascaridoidea							
Ascarididae							
Ophidascaris robertsi (Sprent and Mines 1960)		+					
Spiruroidea							
Gongylonematidae							
Gongylonema sp.		+	+		+		
Spiruridae							
Protospirura marsupialis Baylis 1927		+				+	
Filariodea							
Onchocercidae							
Breinlia trichosuri (Breinl 1913)		+				+	
Sprattia venacavincola (Spratt and Varughese 1975)		+				+	

Endoparasites of *Trichosurus caninus* and locations in which they were recorded

Parasite species	Vic	NSW	Qld
Protozoa			
Eimeriidae			
Eimeria sp.		+	
Sarcocystidae			
Sarcocystis spp.		+	
Cestoda			
Anoplocephalidae			
Bertiella trichosuri Khalil 1970	+	+	
Nematoda			
Rhabditoidea			
Strongyloididae			
Parastrongyloides trichosuri Mackerras 1959		+	
Trichostrongyloidea			
Herpetostongylidae			
Paraustrostrongylus trichosuri Mawson 1973	+	+	
Dromaeostrongylidae			
Filarinema trichosuri (Johnston and Mawson 1939)		+	
Metastrongyloidea			
Angiostrongylidae			
Marsupostrongylus minesi Spratt 1979		+	+
Oxyuroidea			
Adelonema trichosuri (Johnston and Mawson 1938)		+	
Ascaridoidea			
Ascarididae			
Ophidascaris robertsi (Sprent and Mines 1960)		+	
Filariodea			
Onchocercidae			
Breinlia trichosuri (Breinl 1913)		+	
Sprattia venacavincola (Spratt and Varughese 1975)		+	+

Appendix 2:

Small-scale Infection study

Large amounts of parasite material were necessary for protein isolation procedures and for larval and adult culturing An infection programme was designed and implemented, aimed at investigating and overcoming some problems of persistent low levels of infection encountered between December 1997 and 1998. Four parameters were investigated to determine whether these were important in the intensity of infection resulting after experimental infection.

1) Prior exposure to *P. trichosuri*. Two populations of possums were obtained, one from an area where the parasite is endemic, one from an area where *P. trichosuri* is not present so possums would be naïve to this parasite. Possums from Whiteman's Valley, designated the JOTB series, were very young possums weighing less than 2kg that had been exposed to the parasite but, it was hoped, had not yet become immune. The second was from Lewis Pass in the South Island, an area where *P. trichosuri* is not found; these were called the SI possums.
2) Method of infection. In captivity, possums up to the time of the study were usually infected with *P. trichosuri* by subcutaneous injection; this was compared with infection by skin penetration [trans-dermal infection], which is a natural method in the field.
3) Level of infection. Possums were infected with numbers of larvae at what were designated as low [1000 L3/possum], medium [3000-7000 L3/possum], and high levels [>8000 L3/possum], by subcutaneous injection. Subsequent levels of parasitism and duration of infection were monitored. Trans-dermal infection by skin penetration used only high levels of infection similar to work done prior to 1997 [Stankiewicz 1996].
4) Strain of parasite. An observation that parasites which had been passed through several generations in possums in captivity appeared less long lived and robust suggested that a monospecific infection from a wild possum be re-established [Pt98] and compared with existing laboratory cultures [Pt95] for infectivity, levels of infection achieved and length of infection.

Onset, intensity and duration of infection were monitored by standard faecal egg counts.

Results and Discussion:

Initial infection of four possums, infected in June 1997, gave rise to very heavy infections of between 8000-80,000 e.p.g, shown in Table 2.2. It was possible that the infection dose was higher than recorded as methods of larval collection, maintenance and counting were being developed. In particular there were many more non-moving and "pointed" larvae than were observed during later larval collections; these were counted as non-viable but may have been only temporarily non-motile. Better methods in later infection work gave greater confidence in larval counts.

Subsequently, possums had very much lower infections with faecal egg counts never exceeding 150 e.p.g and for some time no possum became heavily parasitized. In some cases faeces with an egg count of zero can be cultured and a significant number of larvae develop. This is particularly true for *P. trichosuri* where the free living stage of the life cycle may allow the parasite to undergo several free-living cycles so increasing numbers before most larvae become infective. Although faeces with low egg counts may give rise to many millions of infective larvae quite sufficient for infecting other possums and maintaining a pure culture, they do not provide a sufficient mass of substance for standard protein purification methods. Therefore the infection study was done to overcome some of the problems of low intensity of infection, this was presumed to be due to immunity as possums have been shown to become immune to artificially induced infection in the laboratory [Heath *et al.*, 1999].

Faecal egg count data from all possums used in this project are shown in Tables 2.2 and 2.3 and in Figs 2.2-.6.

South Island possums have never been exposed to *P. trichosuri* and it was expected that these possums would become heavily infected. Fig 2.3 and Table 2.3 show that only one of seven infected SI possums had an egg count of over 10,000 e.p.g. compared with four from the North Island. From these small numbers, and subsequent work [Ralston, personal communication], it would appear that South Island possums have a similar susceptibility to infection by the parasite as North Island possums. There were no indications that the natural method of infection by skin penetration would produce higher or more persistent infections, see Fig 2.4. Low levels of infection [1000 L3s or

fewer] seem to lead to low levels of parasitism as shown by possum JOTB 7 and SI 6, but this low level did not persist longer than higher levels of infection. Above this infection level, intensity and duration of infection showed very little correlation with levels of infection; see JOTB 3, 6, 8 and the '97 possums in Fig 2.6.

All the possums were young; the JOTB possums were less than one year and the SI possums were thought to be young on the basis of size. Six out of the twelve JOTB possums had initial infections with *P. trichosuri* and *Paraustrostrongylus trichosuri*; this prevalence, 50%, cannot be compared with the prevalence found in other studies [Chapter I.4] because of the restricted and non-random nature of the sample. Previously infected possums [JOTB 1, 2, 3, 6, 7 and 10] did not show lower levels of infection nor was there any difference in the persistence [time to FEC=0] compared with those having no initial infection. Therefore previous infection history did not appear to affect infection in this sample.

These data are considered to be preliminary. They were not treated statistically as the numbers of possums in many of the sub-groups were small, although comparable to other studies with these animals [Viggers *et al.*, 1998, Heath *et al.*, 1999, Ralston *et al.*, 2001a]. Also only young or naïve possums were used and there was no attempt at representative sampling across the population. The chief aim of this study was to overcome problems of possible immunity and determine the characteristics most likely to produce a heavy and/or long lasting infection in order to obtain the very large numbers of larvae and adults necessary for subsequent procedures. From these data it can be seen that none of the factors investigated consistently led to a high or sustained intensity of infection. Other factors, possibly the age and the immunological condition of the individual possum may be more important.

Table A. 2.1 Early Possums - Infection Data

Possum No	Date of Infection	Larval strain	Infection method	Infection dose no. of Pt L3	Incubation-time(wk) to FEC = +ve	Intensity-max FEC e.p.g	Duration-time(wk) till FEC = 0
CA620CT	6/06/1997	Pt95	sci	6000	3	53700	x[3]
34E50DT	6/06/1997	Pt95	sci	6000	?	7900	14
21785FT	6/06/1997	Pt95	sci	6000	3	79000	x[3]
216868T	6/06/1997	Pt95	sci	6000	3	20800	14
3C5A52T	28/01/1998	Pt95	sci	4500	?5	150	8
3C704CT	22/01/1998	Pt95	sci	4500	?8	50	8
ex eprinex 1	22/01/1998	Pt95	sci	4500	?2	150	5

sci= infection by sub-cutaneous injection

e.p.g= eggs per gram

x = FEC +ve at termination
[n] = number of weeks monitored

Table A.2.2 Infection Study Data

Possum No	Possum wt(kg)	Infection history initial FEC.	Infection date re infection date	Larval strain	Infection method	infection dose no. Pt L3	Incubation-time(wk) to positive FEC	Intensity-max FEC e.p.g	Duration-time(wk) till FEC = 0
JOTB 1	1.9	465	6/04/1998	Pt95	sci	8000	2	200	27
JOTB 1			28/05/1998	Pt95	t/d	25000	1	1800	17
JOTB 2	1.55	50	6/04/1998	Pt95	sci	9500	ni	0	
JOTB 2			4/06/1998	Pt98	t/d	25000	3	300	5
JOTB 3	1.7	330	24/03/1998	Pt95	sci	3000	3	16000	x [15]
JOTB 4 bk rd		0	21/07/1998	Pt95	t/d	3000	2	9900	11
JOTB 5	1.35	0	6/04/1998	Pt95	sci	11600	2	3900	16
JOTB 6	1.2	100	1/05/1998	Pt95	sci	5000	3	25000	>24
JOTB 7	1.6	435	1/05/1998	Pt95	sci	1000	3	2250	15
JOTB 7			27/07/1998	Pt95	sci	10,000	ni	0	
JOTB 8	1.5	0	1/05/1998	Pt95	sci	10000	3	20250	10
JOTB 10	1.6	50	28/05/1998	Pt95	t/d	25000	2.5	2850	14
JOTB 11	2.4	0	never infected	neg. control				0	
JOTB 12	2.2	0	never infected	neg. control				0	
JOTB 13	1.9	0	21/07/1998	Pt95	oral	4000	2	780	x[3]
SI 1	1.4-2.0	0	1/05/1998	Pt95	sci	10000	x	died	x[2]
SI 3	1.4-2.0	0	4/06/1998	Pt98	sci	5000	8	630	x[13]
SI 4	1.4-2.0	0	1/05/1998	Pt95	sci	5000	3	630	x [18]
SI 5	1.4-2.0	0	never infected	neg. control				0	
SI 6	1.4-2.0	0	1/05/1998	Pt95	sci	1000	3	2625	7
SI 7	1.4-2.0	0	28/04/1998	Pt98	t/d	13500	2	11250	12
SI 9	1.4-2.0	0	21/07/1998	Pt95	oral	4000	2	270	x [3]
SI 10	1.4-2.0	0	21/07/1998	Pt95	t/d	4000	2	2200	x [3]
SI 11	1.4-2.0	0	never infected	neg. control				0	
SI 12	1.4-2.0	0	24/04/1998	Pt95	sci	5000	4	270	12
			4/06/1998	Pt98	sci	10000	3	130	6

Pt95=infection with existing strain of Pt
Pt98= infection with a new strain of Pt

sci= infection by sub-cutaneous injection
t/d=trans-dermal infection by skin penetration

ni= never infected

x = FEC +ve at termination
[n]=number of weeks monitored

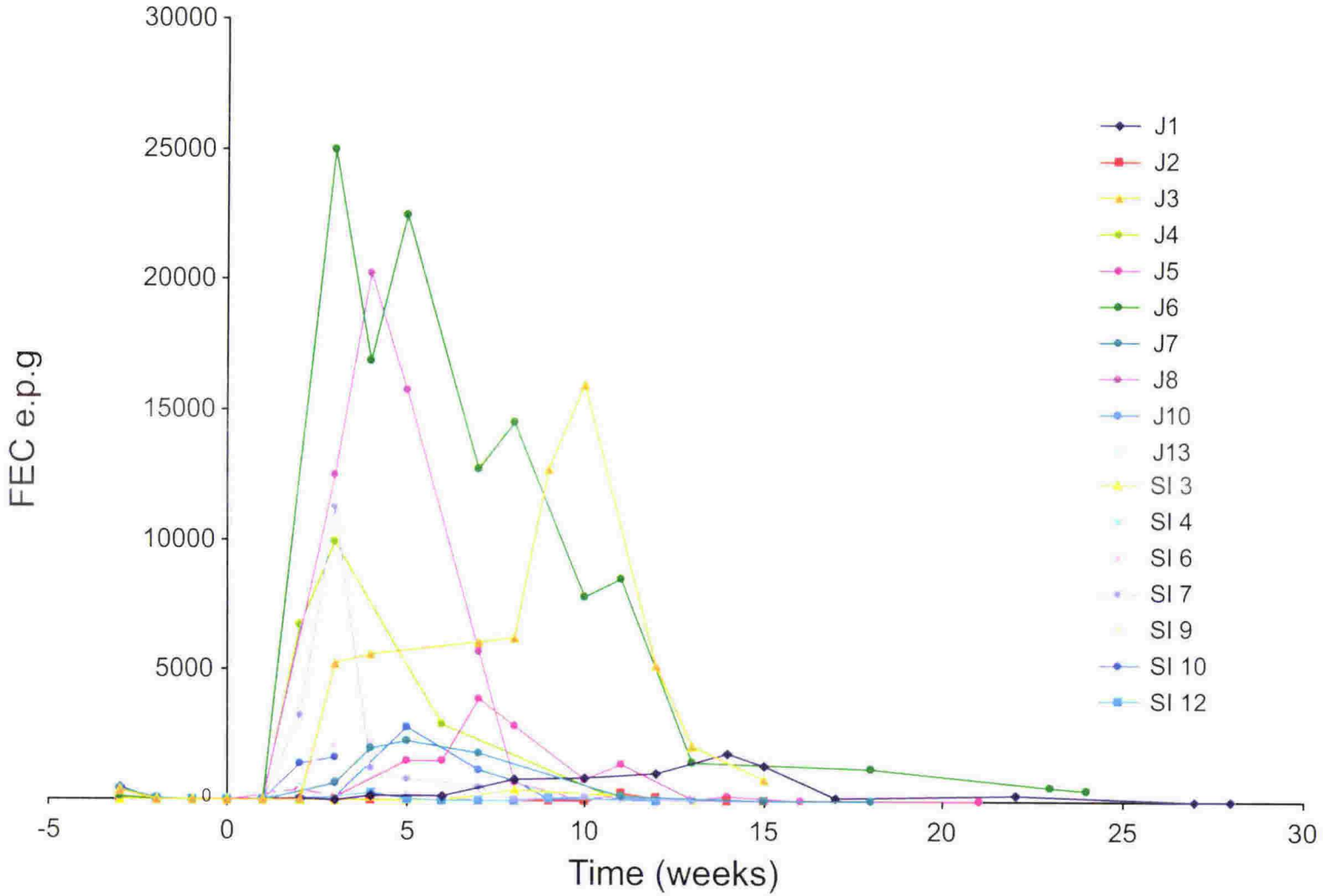
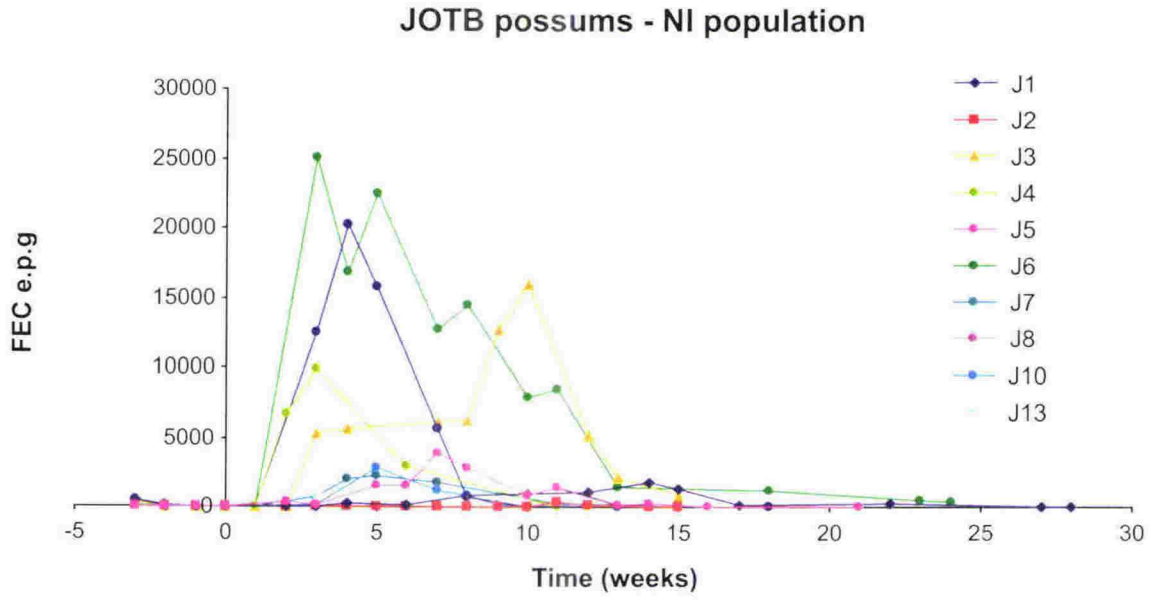


Fig A.2.1 All possums: Infection with *P. trichosuri*.



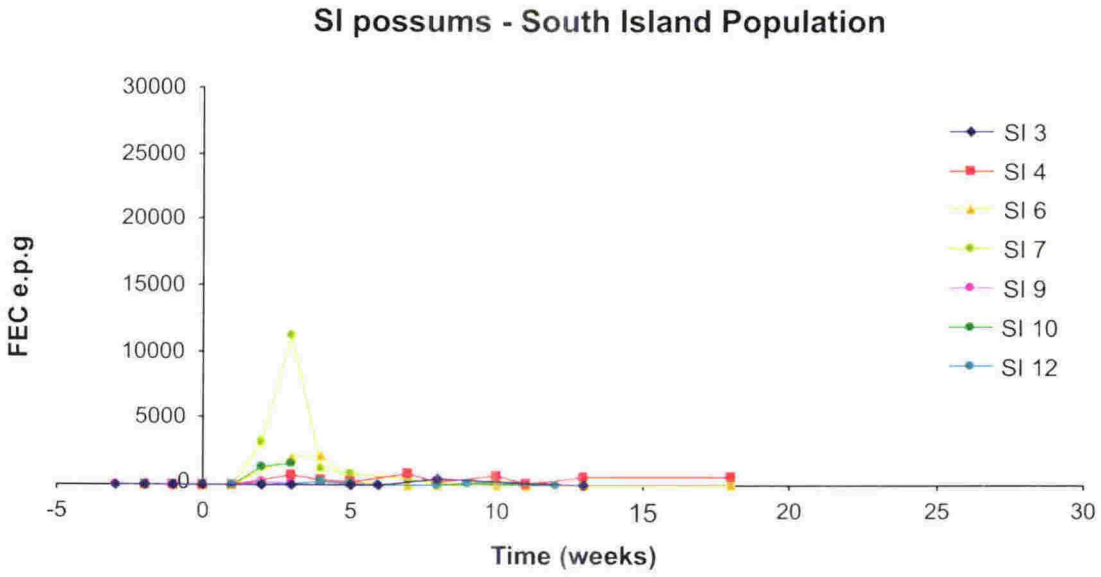


Fig A.2.2 Comparison of infection levels between North Island and South Island possum populations.

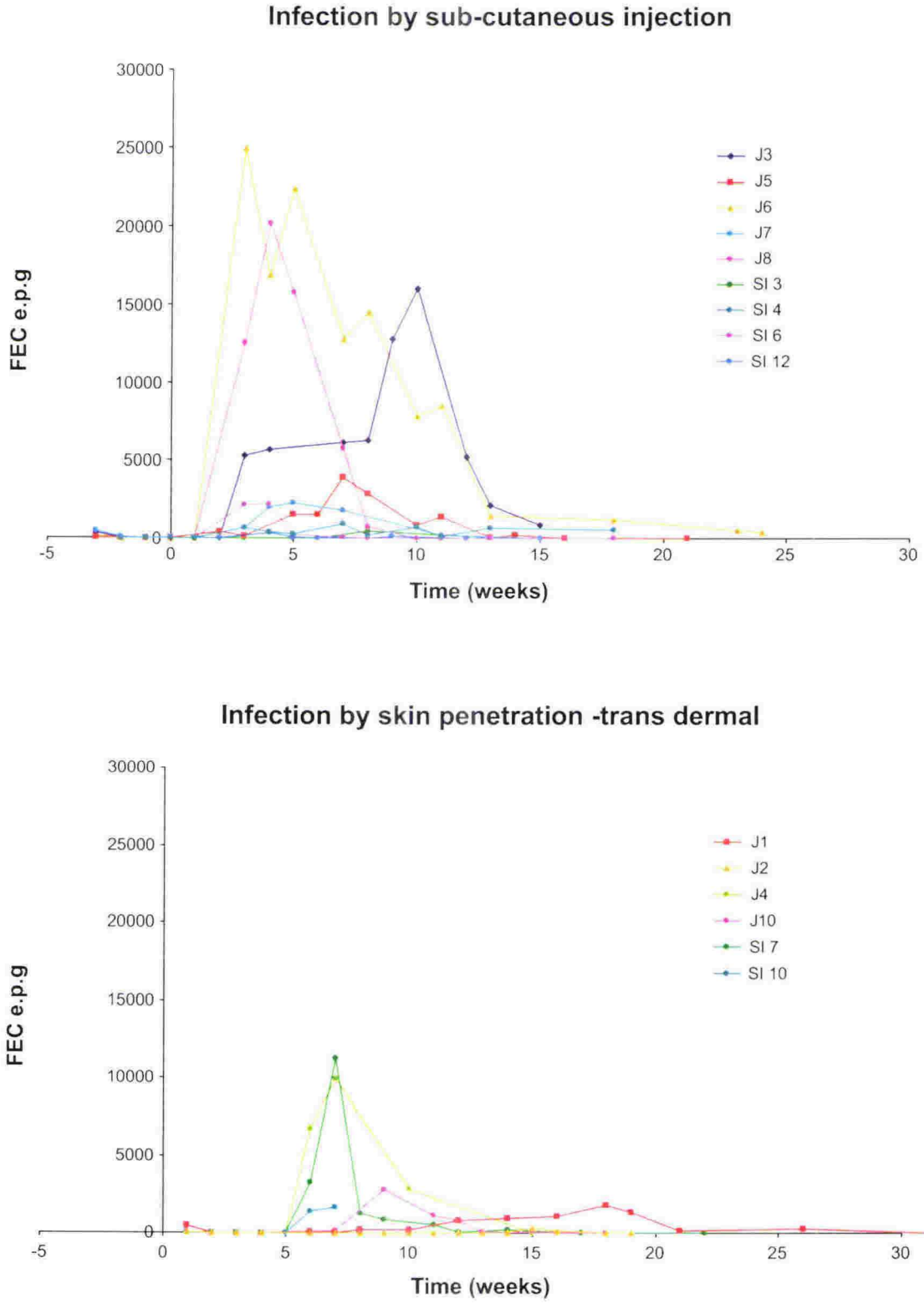


Fig A.2.3 Comparison of infection levels resulting from different methods of infection: either by subcutaneous injection or trans dermally by larval penetration of the skin.

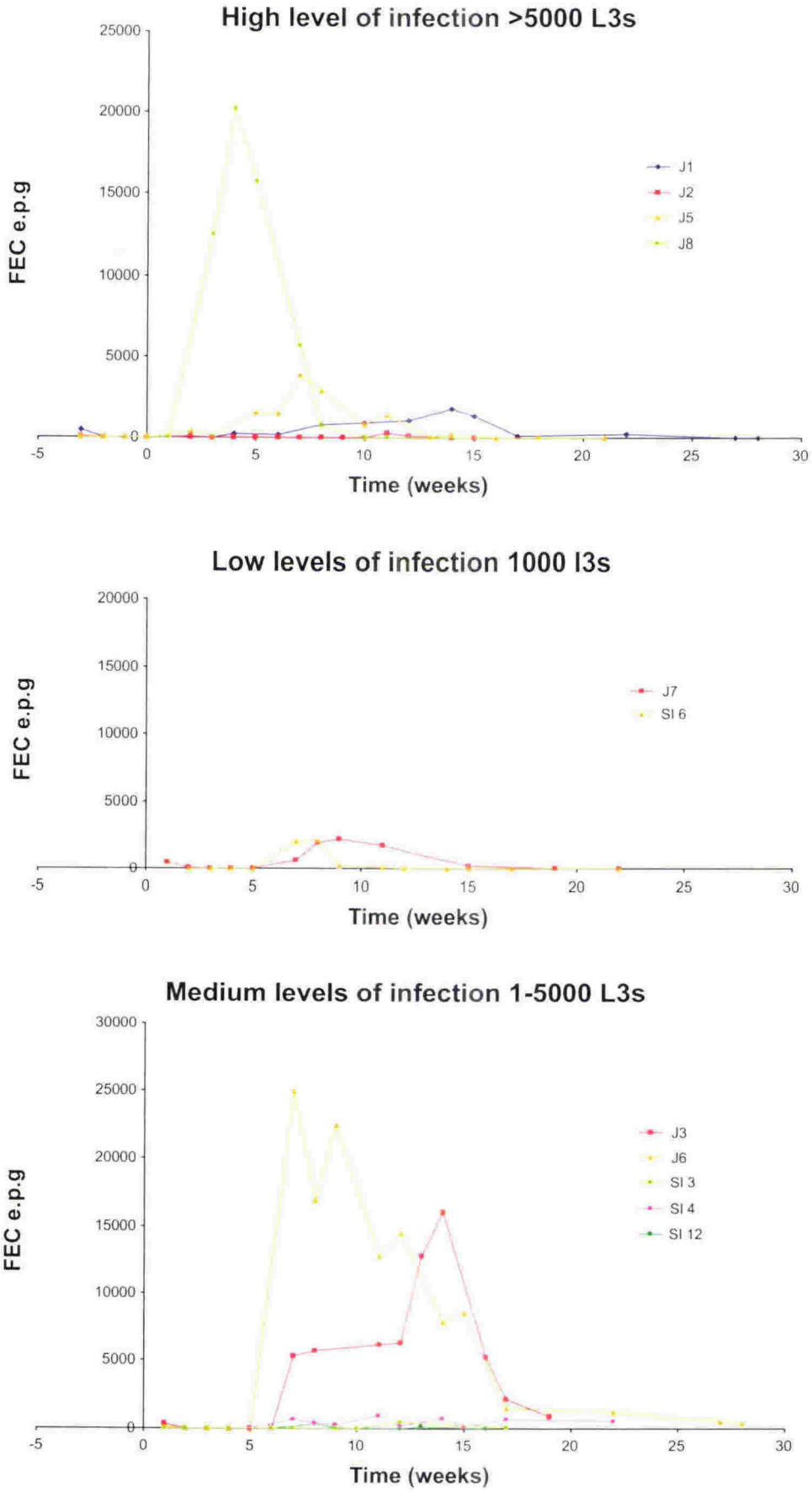
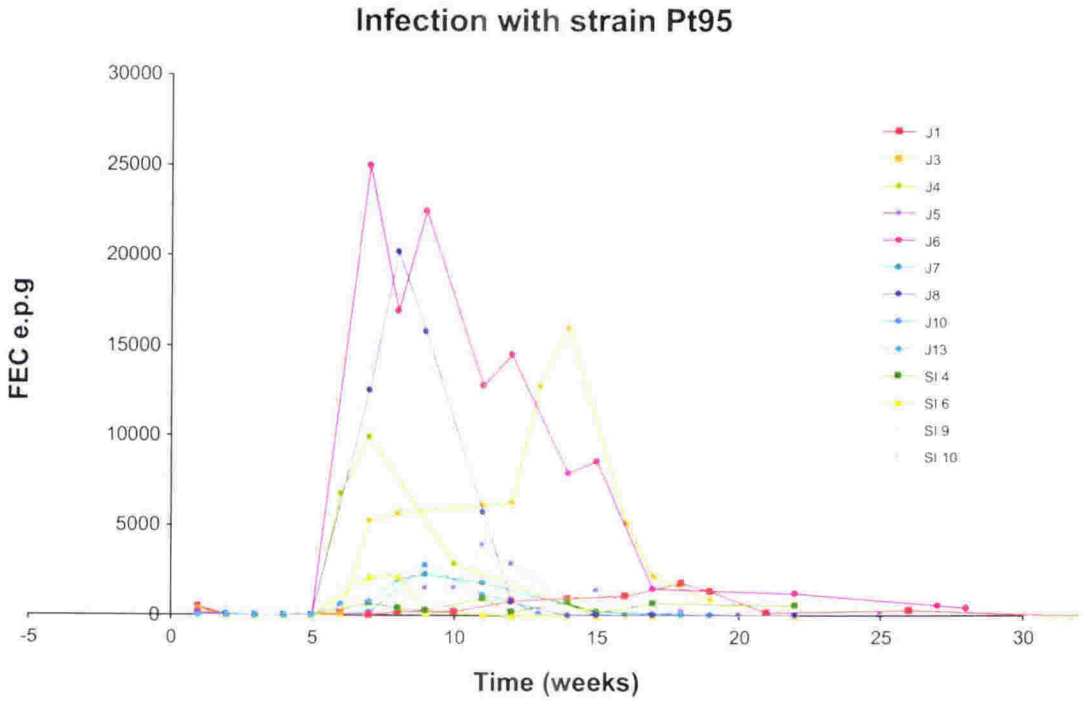


Fig A.2.4 Comparison of infection levels resulting from different doses (number) of infective larvae.



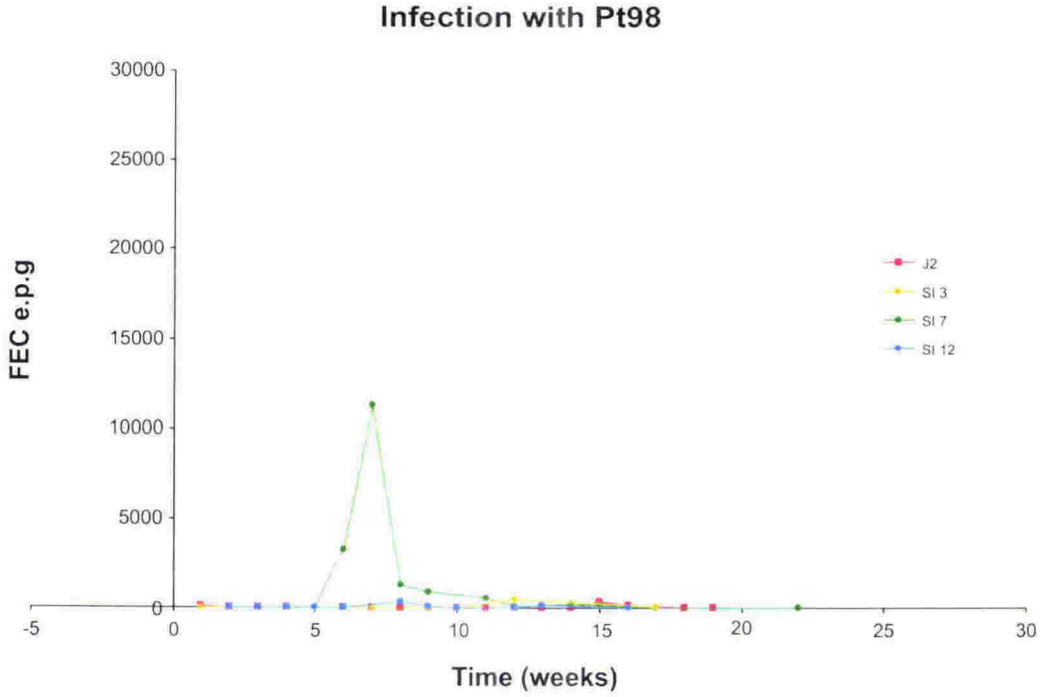


Fig A.2.5 Comparison of infections with two different strains of *P. trichosuri* larvae.

Appendix 3: Solutions

Culture Medium for Parasitic Adult Worms

Ingredient	g/L	
NCTC-135	9.4	Sigma [N3262]
L Cysteine free base	0.25	Sigma [C8152]
Sodium bicarbonate	2.2	
Glucose	3.0	
Penicillin G	0.1	Sigma 1592 u/mg
Streptomycin sulphate	0.1	Sigma 767 u/mg
Gentamycin	0.05	Serva 665 u/mg
Glutamax	3 0	

pH =7.2
filter sterilize

Denhardt's solution:

2% BSA
2% Ficoll
2% polyvinyl pyrrolidine

Hybridization solution:	ml [for 20 ml]
50X Denhardt's solution	2.0
20X SSPE	5.0
10% SDS	0.2
Deionised formamide	10.0
Sonicated salmon sperm DNA [for non homologous DNA]	0.1
Deionized water	2.7

High Salt TBS X1	g/L
Tris base	2.4
Sodium chloride	29.2
Adjust to pH 7.5	
Add 0.05% Thimerosal	

Mouse PBS

	g/L
Sodium chloride	8.0
Potassium chloride	0.2
Potassium dihydrogen phosphate	0.2
Sodium monohydrogen phosphate	0.2
Deionized water to	1L

Adjust to pH 7.2

SSC Buffer x20

	g/L	
Sodium chloride	175.3	
	Sodium citrate	88.2

800ml deionised water
Adjust to pH 7.0 with 10N sodium hydroxide
Add deionised water to a final volume of 1L

SSPE X20

3.6 M sodium chloride
0.2 M sodium dihydrogen phosphate
0.02 M EDTA

Appendix 4:

Multiple Alignment of Nematode Hsp70 Protein Sequences

```
Programme = Pileup [GCG]

       GapWeight: 8
        GapLengthWeight: 2

  Name:                                GenProt accession number:

 Dirofilaria. immitis                          aaa28298
 Brugia malayi                                 aac17926
 Brugia pahangi                                aaa27857
 Wucheria bancrofti                            aaf66987
 Setaria digitata                              aad13154
 Parastrongyloides trichosuri                  aaf87583
 Onchocerca volvulus                           aaa29417
 Caenorhabditis elegans                        aaa28078
 Onchocerca volvulus                           aab30260
 Heterodera glycines                           aag47839
 Trichinella britovi                           caa73574

//

                1                                                        50
D. immitis      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
B. malayi       ~~MSKNAIGI DLGTTYSCVG VFMHGKVEII ANDQGNRTTP SYVAFTDTER
B. pahangi      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
W. bancrofti    ~~MSKNAIGI DLGTTYSWVG VFMHGKVEII ANDQGNRTTP SYVAFTDTER
S. digitata     ~~MSKNAIGI DLGTTYSCVG VFMHGKVEII ANDQGNRTTP SYVAFTDTER
P. trichosuri   ~MVKANAIGI DLGTTYSCVG VFQHGKVEII ANDQGNRTTP SYVAFTDSER
O. volvulus-2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
C. elegans      ~MSKHNAVGI DLGTTYSCVG VFMHGKVEII ANDQGNRTTP SYVAFTDTER
O. volvulus-1   ~MGKSNAVGI DLGTTYSCVG VFMHGKVEII ANDQGNRTTP SYVAFTDTER
H. glycines     MPSKANAIGI DLGMTYSCVG VFQHGKVEII ANDQGNRTTP SYVAFTDTER
T. britovi      ~MSK.NAIGI DLGTTYSCVG VFQHGKVEII ANDQGNRTTP SYVAFTDTER

                51                                                       100
D. immitis      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
B. malayi       LIGDAAKNQV AMNPHNTVFD ANRLIGRKFD DGSVQSDMKH WPFKVVNAGG
B. pahangi      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
W. bancrofti    LIGDAAKNQV AMNPHNTVFD AKRLIGRKFD DGSVQSDMKH WPFKVVNAGG
S. digitata     LIGDAAKNQV AMNPHNTVFD AKRLIGRKFD DGSVQSDMKH WPFKVMNAGG
P. trichosuri   LIGDAAKNQV AMNPQNSVFD AKRLIGRKFD EAAVQADMKH WPFKVISADG
O. volvulus-2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
C. elegans      LIGDAAKNQV AMNPHNTVFD AKRLIGRKFD DPAVQSDMKH WPFKVISAEG
O. volvulus-1   LIGDAAKNQV AMNPHNTVFD AKRLIGRKFD DPAVQSDMKH WPFKVI~~~~
H. glycines     LIGDAAKNQV AMNPSNTVFD AKRLIGRKFD DPAVQSDMKH WPFKVVQGEG
T. britovi      LIGDAQKNQV ALNPHNTVFD AKRLIGRRFD DAAVQSDMKH WPFKIIN.DG

                101                                                      150
D. immitis      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
B. malayi       GKPKVQVEYK GETKTFTPEE ISSMVLVKMK ETAEAFLGHA VKDAVITVPA
B. pahangi      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
W. bancrofti    GKPKVQVEYK GETKTFTPEE ISSMVLVKMK ETAEAFLGHA VKDAVITVPA
S. digitata     GKPKVQVEYK GETKTFTPGE ISSMVLVKMK ETAEAFLGHA VKDAVITVPA
P. trichosuri   GRPKVQVEFK GEVKTFFPEE ISSMILTKMR ETAEAYLGQT VTDAVVTVPA
O. volvulus-2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
C. elegans      AKPKVQVEYK GENKIFTPEE ISSMVLLKMK KTAEAFLEPT VKDAVVTVPT
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     ARPKVMVEVK GEDKAFFPEE VSSMVLIKMK ETAEAFLGQT VTDAVVTVPA
T. britovi      SKPKIQVEYK GESKSFTPEE ISAMVLVKMK ETAEAYLGKT VKDAVITVPA

                151                                                      200
D. immitis      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
```

```
B. malayi       YFNDSQRQAT KDSGAIAGLN VLRIINEPTA AAIAYGLDKK GHGERNVLIF
B. pahangi      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
W. bancrofti    YFNDSQRQAT KDSGAIAGLN VLRIINEPTA AAIAYGLDKK GHGERNVLIF
S. digitata     YFNDSQRQAT KDSGAIAGLN VLRIINEPTA AAIAYGLDKK GHGERNVLIF
P. trichosuri   YFNDSQRQAT KDAGAIAGLN VLRIINEPTA AAIAYGLDKK GVSERNVLIF
O. volvulus-2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
C. elegans      YFNDSQRQAT KDAGAIAGLN VLRIINEPTA AAIAYGLDKK GHGERNVLIF
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     YFNDSQRQAT KDAGTIAGLN VLRIINEPTA AAIAYGLDKK GQGERHVLIF
T. britovi      YFNDSQRQAT KDAGTISGLN VLRIINEPTA AAIAYGLDRK GGGERNVLIF

                201                                                 250
D. immitis      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
B. malayi       DLGGGTFDVS ILTIEDGIFE VKSTAGDTHL .GEDFDNRMV NHFVAEFKRN
B. pahangi      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
W. bancrofti    DLGGGTFDVS ILTIEDGIFE VKSTAGDIHL GGEDFDNRMV NHFVAEFKRK
S. digitata     DLGGGTFDVS ILTIEDGIFE VKSTAGDTHL GGEDFDNRMV NHFVAEFKRK
P. trichosuri   DLGGGTFDVS ILTIEDGIFE VKSTAGDTHL GGEDFDNRMV THFVNEFKRK
O. volvulus-2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~EFKRN
C. elegans      DLGGGTFDVS ILTIEDGIFE VKSTAGDTHL GGEDFDNRMV NHFCAEFKRK
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     DLGGGTFDVS ILTIEDGIFE VKSTAGDTHL GGEDFDNRMV NHFVAEFKRK
T. britovi      DLGGGTFDVS ILTIEDGIFE VKSTAGDTHL VGEDFDNRMV NHFVAEFKRK

                251                                                 300
D. immitis      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
B. malayi       DKKDLASNPR ALRRLRTACE RAKRTLSSSS QASIEIDSLF EGIDFYTNIT
B. pahangi      ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
W. bancrofti    HKKDLASNLR ALRRLRTACE RAKRTLSSSS QASIEIDSLF EGIDFYTNIT
S. digitata     HKKDLASNPR ALRRLRTACE RAKRTLSSSS QASIEIDSLF EGIDFYTNIT
P. trichosuri   HKKDLSANPR ALRRLRTACE RAKRTLSSST QASIEIDSLF DGIDFYTNIT
O. volvulus-2   DKKDLASNPR ALRRLRTACE RAKRTLSSSS QASIEIDSLF EGIDFYTNIT
C. elegans      HKKDLASNPR ALRRLRTACE RANETLSSSC QASIEIDSLF EGIDFYTNIT
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     HKKDLSSNPR ALRRLRTACE RAKRTLSGST QASIEIDSLF DGIDFYTNIT
T. britovi      NKKDMSSNPR ALRRLRTACE RAKRTLSSST QASIEIDSLY EGIDFYTTIT

                301                                                 350
D. immitis      ~~RFEELCAD LFRSTMDPVE KALRDAKMDK AQVHDIVLVG GSTRIPKVQK
B. malayi       RARFEELCAD LFRSTMDPVE KALRDAKMDK AQVHDIVLVG GSTRIPKVQK
B. pahangi      ~~~~~~~~~~ ~~LSTMDPVE KALRDAKMDK AQVHDIVLVG GSTRIPKVQK
W. bancrofti    RARFEELCAD LFRSTMDPVE KALRDAKMDK AQVHDIVLVG GSTRIPKVQK
S. digitata     RARFEELCAD LFRSTMDPVE KALRDAKMDK AQVHDIVLVG GSTRIPKVQK
P. trichosuri   RARFEELCAD LFRNTMDPVE KALRDAKMDK GQIHDIVLVG GSTRIPKVQK
O. volvulus-2   RARFEELCAD LFRSTMDPVE KALRVAKMDK AQVHDIVLVG GSTRIPKVQK
C. elegans      RARFEELCAD LFRSTMDPVE KSLRDAKMDK SQVHDIVLVG GSTRIPKVQK
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     RARFEELCAD LFRNTMDPVE KALRDAKMDK SQIHDIVLVG GSTRIPKVQK
T. britovi      RARFEELNAD LFRSTLEPVE KALRDAKLDK AVIHEVVLVG GSTRIPKVQK

                351                                                 400
D. immitis      LLSDFFSGKE LNKSINPDEA VAYGAAVQAA ILSGDKSEAV QDLLLLDVAP
B. malayi       LLSDFFSGKE LNKSINPDEA VAYGAAVQAA ILSGDKSEAV QDLLLLDVAP
B. pahangi      LLSDFFSGKE LNKSINPDEA VAYGAAVQAA ILSGDKSEAV QDLLLLDVAP
W. bancrofti    LLSDFFSGKE LNKSINPDEA VAYGAAVQAA ILSGDKSEAV QDLLLLDVAP
S. digitata     LLSDFFSGKE LNKSINPDEA VAYGAAVQAA ILSGDKSEAV QDLLFVDVAP
P. trichosuri   LLSDFFSGKE LNKSINPDEA VAYGAAVQAA ILSGDKSEAV QDLLLLDVAP
O. volvulus-2   LLSDFFSGKE LNKSINPDEA VAYGAAVQAA ILSGDKSEAV QDLLLLDVAP
C. elegans      LLSDLFSGKE LNKSINPDEA LAYGAAVQAA ILSGDKSEAV QDLLLLDVAP
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     LLSDFFSGKE LNKSINPDEA VAYGAAVQAA ILSGDKSETV QDLLLLDVAP
T. britovi      LLQDFFNGKE LNKSINPDEA VAYGAAVQAA ILSGEKHEAV QDLCLLDVTP

                401                                                 450
D. immitis      LSLGIETAGG VMTALIKRNT TIPTKTSQTF TTYSDNQPGV LIQVYEGERA
B. malayi       LSLGIETAGG VMTALIKRNT TIPTKTSETF TTYSDNQPGV LIQVYEGERA
B. pahangi      LSLGIETAGG VMTALIKRNT TIPTKTSQTF TTYSDNQPGV LIQVYEGERA
W. bancrofti    LSLGIETAGG VMTALIKRNT TIPTKTSQTF TTYSDNQPGV LIQVYEGERA
S. digitata     LSLGIETAGG VMTALIKRNT TIPTKTSQTF TTYSDNQPGV LIQVYEGERA
P. trichosuri   LSLGIETAGG VMTALIKRNT TIPTKTSQTF TTYADNQPGV LIQVYEGERA
```

```
O. volvulus-2   LSLGIETAGG VMTALIKRNT TIPTKTSQTF TTYSDNQPGV LIQVYEGERA
C. elegans      LSLGIETAGG VMTALIKRNT TIPTKTAQTF TTYSDNQPGV LIQVYEGERA
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     LSLGIETAGG VMTSLIKRNT TIPTKTSQTF TTYSDNQPGV LIQVYEGERA
T. britovi      LSLGIETAGG VMTALIKRNT TIPTKVSQVF TTYSDNQPGV LIQVYEGERA

                451                                                 500
D. immitis      MTKDNNLLGK FELSGIPPAP RGVPQIEVTF DIDANGILNV SAQDKSTGKQ
B. malayi       LTKDNNLLGK FELSGIPPAP RGVPQIEVTF DIDANGILNV SAQDKSTGKQ
B. pahangi      LTKDNNLLGK FELSGIPPAP RGVPEIEVTF DIDANGILNV SAQDKSTGKQ
W. bancrofti    LTKDNNLLGK FELSGIPPAP RGVPQIEVTF DIDANGILNV SAQDKSTGKQ
S. digitata     MTKDNNLLGK FELSGIPPAP RGVPQIEVTF DIDANGILNV SAQDKSTGKQ
P. trichosuri   MTKDNNLLGK FELSGIPPAP RGVPQIEVTF DIDANGILNV SAQDKSTGKQ
O. volvulus-2   MTKDNNLLGK FELSGIPPAP RGVPQIEVTF DIDANGILNV SAQDKSTGKQ
C. elegans      MTKDNNLLGK FELSGIPPAP RGVPQIEVTF DIDANGILNV SATDKSTGKA
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     MTKDNNLLGK FELSGIPPAP RGVPQIEVTF DIDANGILNV SAQDKSTGKQ
T. britovi      MTKDNNLLGK FELTGIPPAP RGVPQIEVTF DIDANGILNV SAVDKSTGRQ

                501                                                 550
D. immitis      NKITITNDKG RLSKDEIERM VQEAEKYKAD DEAQKDRIAA KNALESYAFN
B. malayi       NKITITNDKG RLSKDEIERM VQEAEKYKAD DEAQKDRIAA KNALESYAFN
B. pahangi      NKITITNDKG RLSKDEIERM VQEAEKYKAD DEAQKDRIAA KNALESYAFN
W. bancrofti    NKITITNDKG RLSKDEIERM VQEAEKYKAD DEAQKDRIAA KNALESYAFN
S. digitata     NKITITNDKG RLSKDEIERM VQEAEKYKAD DEAQKDRIAA KNALESYAFN
P. trichosuri   NKITITNDKG RLSKEEIERM VNDAEKYKAD DEKQRDRVAA KNGLESYCFN
O. volvulus-2   NKITITNDKG RLSKDEIERM VQEAEKYKAD DEAQKDRIAA KNALESYAFN
C. elegans      KQITITNDKD RFSKDDIERM VNEAEKYKAD DEAQKDRIGA KNGLESYAFN
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     NKITITNDKG RLSKEEIERM VQEAEKFSKE DEVQRDRVSA KNALESYCFN
T. britovi      NKITITNDKG RLSKEDIDRM VREADQYKQE DEKQRDRIQA KNGLESYAFN

                551                                                 600
D. immitis      MKQTIEDEKL KDKISEDDKK KIQEKCDETV RWLDGNQTAE KDEFEHRQKE
B. malayi       MKQTIEDEKL KDKISEEDKK KIQEKCDETV RWLDGNQTAE KDEFEHRQKE
B. pahangi      MKQTIEDEKL KDKISEEDKK KIQEKCDETV RWLDGNQTAE KDEFEHRQKE
W. bancrofti    MKQTIEDEKF KDKISEEDKK KIPEKCDETV TWLDGNQTAE KDEFEHRQKE
S. digitata     MKQTIEDEKL RDKLSEEDKK KIQEKCDETV RWLDGNQTAE KDEFEHRQKE
P. trichosuri   MKQTLEDEKV KDKIPADDAK KVLDKCDEVL KWLDSNQSAE KEEFEDRQKE
O. volvulus-2   MKQTIEDEKL KDKISEF~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
C. elegans      LKQTIEDEKL KDKISPEDKK KIEDKCDEIL KWLDSNQTAE KEEFESQQKD
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     IKQTMEDSNL KDKISEDDKK KVLEKCGEVL AWLDANQAAE KEEFEHQQKE
T. britovi      VKSTIEDEKL KDKIPESDRK AVLNKCEEVL RWLETNQLAE KDEFEHKQKD

                601                                                 650
D. immitis      LESVCNPIIT KLYQSAGGMP GG.MPGGMPG GAPGGGSTG. .GGPTIEEVD
B. malayi       LESVCNPIIT KLYQSAGGMP GG.MPGGMPG GAPGAGSTG. .GGPTIEEVD
B. pahangi      LESVCNPIIT KLYQSAGGMP GG.MPGGMPG GAPGAGSTG. .GGPTIEEVD
W. bancrofti    LESVCNPIIT KLYQSAGGMP GG.MPGGMPS GAPGAGSTG. .GGPTIEEVD
S. digitata     LEAVSNPIIT KLYQSAGGMP GG.MPGGMPG GAPGGGSGG. .SGPTIEEVD
P. trichosuri   LEGICNPIIT KMYGAAGGPP GG.MPGGAPG GAPGG..EG. .SGPTIEEVD
O.  volvulus-2          ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~
~~~~~~~~~~
C. elegans      LEGLAKPDLS KLYQSAGGAP PGAAPGG... ......AAGG AGGPTIEEVD
O. volvulus-1   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
H. gylcines     LEGICNPIIT KLYQAGGAMP GGPMPGGGMP GGGGAAGAGG AGGPTIEEVD
T. britovi      LESLCNP.IC KCIRMRVNAS GMPNLQFLQV DLQVASSRGG GGGPTIEEVD
```

Appendix 4:

Comparison of the genomic sequence for PtHsp70 with the cDNA sequence for the Pt Hsp70 gene using the GAP programme [GCG]. There is one intron shown from position 109 to 151 .

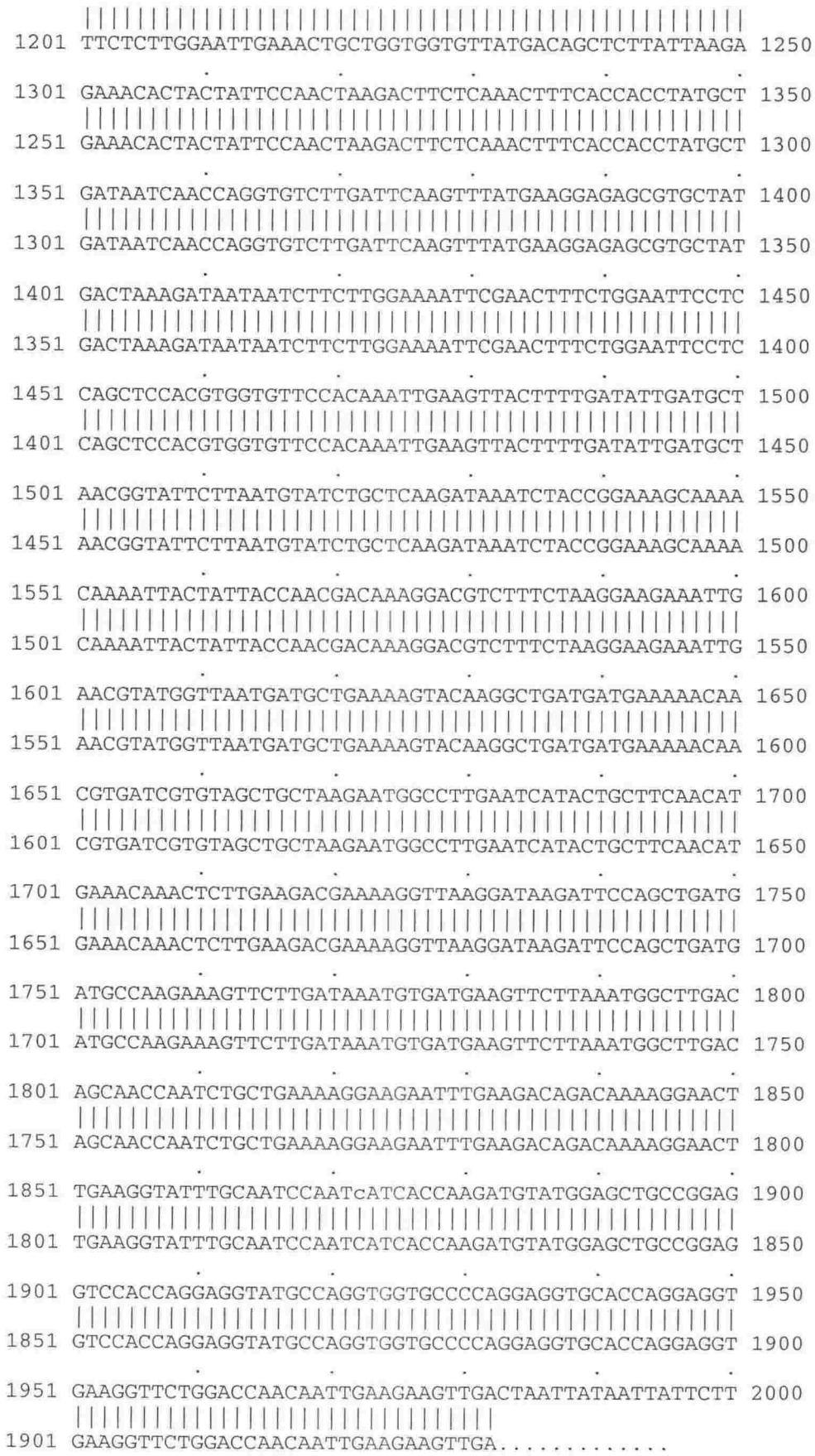
```
GAP of: Ptgencoding.seq
     to: PtcDNAcoding.con

 Gaps:1   Percent Similarity: 99.948   Percent Identity: 99.948

   Match display thresholds for the alignment(s):
              | = IDENTITY
              : =   5
              . =   1


            .         .         .         .         .
  1 AAGAAAAAATGGTTAAAGCTAATGCTATCGGTATTGATCTCGGTACCACA 50
            |||||||||||||||||||||||||||||||||||||||||||
  1 .......TATGGTTAAAGCTAATGCTATCGGTATTGATCTCGGTACCACA 43
            .         .         .         .         .
 51 TATTCATGTGTAGGTGTTTTCCAACATGGAAAAGTTGAAATTATTGCTAA 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 44 TATTCATGTGTAGGTGTTTTCCAACATGGAAAAGTTGAAATTATTGCTAA 93
            .         .         .         .         .
101 TGACCAAGGTATGTTTTAGAATTATTATTTTAAAATAATCATTATTTTTA 150
    ||||||||
 94 TGACCAAG..................................... 100
            .         .         .         .         .
151 GGTAATCGTACAACACCATCATACGTTGCCTTCACTGACAGTGAAAGACT 200
     ||||||||||||||||||||||||||||||||||||||||||||||||||
101 .GTAATCGTACAACACCATCATACGTTGCCTTCACTGACAGTGAAAGACT 150
            .         .         .         .         .
201 TATTGGAGATGCTGCCAAAAATCAAGTTGCTATGAACCCACAAAACTCTG 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 TATTGGAGATGCTGCCAAAAATCAAGTTGCTATGAACCCACAAAACTCTG 200
            .         .         .         .         .
251 TCTTTGATGCTAAACGTCTTATTGGAAGAAAATTCGACGAAGCTGCTGTT 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 TCTTTGATGCTAAACGTCTTATTGGAAGAAAATTCGACGAAGCTGCTGTT 250
            .         .         .         .         .
301 CAAGCTGACATGAAACATTGGCCATTCAAAGTTATATCTGCTGATGGAGG 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 CAAGCTGACATGAAACATTGGCCATTCAAAGTTATATCTGCTGATGGAGG 300
            .         .         .         .         .
351 ACGTCCAAAGGTCCAAGTTGAATTCAAAGGAGAAGTCAAGACATTCTTCC 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 ACGTCCAAAGGTCCAAGTTGAATTCAAAGGAGAAGTCAAGACATTCTTCC 350
            .         .         .         .         .
401 CAGAAGAAATTTCATCTATGATCCTTACTAAAATGAGAGAAACTGCTGAA 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 CAGAAGAAATTTCATCTATGATCCTTACTAAAATGAGAGAAACTGCTGAA 400
            .         .         .         .         .
451 GCTTATCTTGGACAAACTGTTACTGATGCTGTTGTCACTGTTCCAGCTTA 500
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 GCTTATCTTGGACAAACTGTTACTGATGCTGTTGTCACTGTTCCAGCTTA 450
            .         .         .         .         .
```

```
501 TTTCAACGACTCTCAAAGACAAGCTACAAAAGATGCTGGAGCCATTGCTG 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
451 TTTCAACGACTCTCAAAGACAAGCTACAAAAGATGCTGGAGCCATTGCTG 500
          .         .         .         .         .
551 GTCTTAATGTTTTACGTATTATTAATGAACCAACAGCCGCTGCTATTGCT 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
501 GTCTTAATGTTTTACGTATTATTAATGAACCAACAGCCGCTGCTATTGCT 550
          .         .         .         .         .
601 TATGGTTTAGATAAGAAAGGAGTTTCTGAACGTAATGTTCTTATTTTTGA 650
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
551 TATGGTTTAGATAAGAAAGGAGTTTCTGAACGTAATGTTCTTATTTTTGA 600
          .         .         .         .         .
651 TCTTGGTGGTGGTACTTTTGATGTATCAATTCTTACTATTGAAGATGGAA 700
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
601 TCTTGGTGGTGGTACTTTTGATGTATCAATTCTTACTATTGAAGATGGAA 650
          .         .         .         .         .
701 TTTTTGAAGTTAAGTCAACTGCTGGAGATACTCATTTAGGAGGTGAAGAT 750
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
651 TTTTTGAAGTTAAGTCAACTGCTGGAGATACTCATTTAGGAGGTGAAGAT 700
          .         .         .         .         .
751 TTTGATAACCGTATGGTTACTCACTTTGTTAATGAGTTTAAAAGAAAGCA 800
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
701 TTTGATAACCGTATGGTTACTCACTTTGTTAATGAGTTTAAAAGAAAGCA 750
          .         .         .         .         .
801 TAAGAAAGATTTATCAGCCAATCCACGTGCTCTCCGTCGTTTAAGAACTG 850
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
751 TAAGAAAGATTTATCAGCCAATCCACGTGCTCTCCGTCGTTTAAGAACTG 800
          .         .         .         .         .
851 CTTGTGAACGTGCTAAGAGAACACTTTCTTCATCAACTCAAGCATCAATT 900
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
801 CTTGTGAACGTGCTAAGAGAACACTTTCTTCATCAACTCAAGCATCAATT 850
          .         .         .         .         .
901 GAAATTGATTCACTTTTTGATGGAATTGATTTCTACACCAACATTACTCG 950
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
851 GAAATTGATTCACTTTTTGATGGAATTGATTTCTACACCAACATTACTCG 900
          .         .         .         .         .
951 TGCTCGTTTTGAAGAACTTTGTGCTGATCTTTTTAGAAATACAATGGATC 1000
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
901 TGCTCGTTTTGAAGAACTTTGTGCTGATCTTTTTAGAAATACAATGGATC 950
          .         .         .         .         .
1001 CAGTTGAAAAAGCTCTTCGTGATGCTAAGATGGACAAAGGACAAATTCAC 1050
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 951 CAGTTGAAAAAGCTCTTCGTGATGCTAAGATGGACAAAGGACAAATTCAC 1000
          .         .         .         .         .
1051 GATATTGTTCTTGTTGGAGGATCAACCCGTATTCCAAAAGTTCAAAAACT 1100
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1001 GATATTGTTCTTGTTGGAGGATCAACCCGTATTCCAAAAGTTCAAAAACT 1050
          .         .         .         .         .
1101 TCTTTCTGATTTCTTCTCTGGAAAGGAACTTAACAAATCTATCAATCCTG 1150
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1051 TCTTTCTGATTTCTTCTCTGGAAAGGAACTTAACAAATCTATCAATCCTG 1100
          .         .         .         .         .
1151 ATGAAGCTGTAGCTTATGGTGCTGCTGTTCAAGCTGCTATTCTTTCAGGA 1200
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1101 ATGAAGCTGTAGCTTATGGTGCTGCTGTTCAAGCTGCTATTCTTTCAGGA 1150
          .         .         .         .         .
1201 GACAAATCTGAAGCTGTCCAAGATTTACTTCTTCTTGATGTCGCTCCACT 1250
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1151 GACAAATCTGAAGCTGTCCAAGATTTACTTCTTCTTGATGTCGCTCCACT 1200
          .         .         .         .         .
1251 TTCTCTTGGAATTGAAACTGCTGGTGGTGTTATGACAGCTCTTATTAAGA 1300
```


1201 TTCTCTTGGAATTGAAACTGCTGGTGGTGTTATGACAGCTCTTATTAAGA 1250
1301 GAAACACTACTATTCCAACTAAGACTTCTCAAACTTTCACCACCTATGCT 1350
1251 GAAACACTACTATTCCAACTAAGACTTCTCAAACTTTCACCACCTATGCT 1300
1351 GATAATCAACCAGGTGTCTTGATTCAAGTTTATGAAGGAGAGCGTGCTAT 1400
1301 GATAATCAACCAGGTGTCTTGATTCAAGTTTATGAAGGAGAGCGTGCTAT 1350
1401 GACTAAAGATAATAATCTTCTTGGAAAATTCGAACTTTCTGGAATTCCTC 1450
1351 GACTAAAGATAATAATCTTCTTGGAAAATTCGAACTTTCTGGAATTCCTC 1400
1451 CAGCTCCACGTGGTGTTCCACAAATTGAAGTTACTTTTGATATTGATGCT 1500
1401 CAGCTCCACGTGGTGTTCCACAAATTGAAGTTACTTTTGATATTGATGCT 1450
1501 AACGGTATTCTTAATGTATCTGCTCAAGATAAATCTACCGGAAAGCAAAA 1550
1451 AACGGTATTCTTAATGTATCTGCTCAAGATAAATCTACCGGAAAGCAAAA 1500
1551 CAAAATTACTATTACCAACGACAAAGGACGTCTTTCTAAGGAAGAAATTG 1600
1501 CAAAATTACTATTACCAACGACAAAGGACGTCTTTCTAAGGAAGAAATTG 1550
1601 AACGTATGGTTAATGATGCTGAAAAGTACAAGGCTGATGATGAAAAACAA 1650
1551 AACGTATGGTTAATGATGCTGAAAAGTACAAGGCTGATGATGAAAAACAA 1600
1651 CGTGATCGTGTAGCTGCTAAGAATGGCCTTGAATCATACTGCTTCAACAT 1700
1601 CGTGATCGTGTAGCTGCTAAGAATGGCCTTGAATCATACTGCTTCAACAT 1650
1701 GAAACAAACTCTTGAAGACGAAAAGGTTAAGGATAAGATTCCAGCTGATG 1750
1651 GAAACAAACTCTTGAAGACGAAAAGGTTAAGGATAAGATTCCAGCTGATG 1700
1751 ATGCCAAGAAAGTTCTTGATAAATGTGATGAAGTTCTTAAATGGCTTGAC 1800
1701 ATGCCAAGAAAGTTCTTGATAAATGTGATGAAGTTCTTAAATGGCTTGAC 1750
1801 AGCAACCAATCTGCTGAAAAGGAAGAATTTGAAGACAGACAAAAGGAACT 1850
1751 AGCAACCAATCTGCTGAAAAGGAAGAATTTGAAGACAGACAAAAGGAACT 1800
1851 TGAAGGTATTTGCAATCCAATcATCACCAAGATGTATGGAGCTGCCGGAG 1900
1801 TGAAGGTATTTGCAATCCAATCATCACCAAGATGTATGGAGCTGCCGGAG 1850
1901 GTCCACCAGGAGGTATGCCAGGTGGTGCCCCAGGAGGTGCACCAGGAGGT 1950
1851 GTCCACCAGGAGGTATGCCAGGTGGTGCCCCAGGAGGTGCACCAGGAGGT 1900
1951 GAAGGTTCTGGACCAACAATTGAAGAAGTTGACTAATTATAATTATTCTT 2000
1901 GAAGGTTCTGGACCAACAATTGAAGAAGTTGA.....

References:

Manual of veterinary parasitological laboratory techniques, (1973). 3rd edition. Her Majesty's Stationery Office, London.

The *C. elegans* Sequencing Consortium, (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282,** 2012-8.

AALFS, J. D. & KINGSTON, R. E. (2000). What does 'chromatin remodeling' mean? *Trends in Biochemical Sciences* **25,** 548-555.

ADAMSKI, F. M. & DEMMER, J. (1999). Two stages of increased IgA transfer during lactation in the marsupial, *Trichosurus vulpecula* (brushtail possum). *Journal of Immunology* **162,** 6009-6015.

ALLEN, A. B. & ROSE, A. B. (1983). Regeneration of southern rata (*Metrosideros umbellata*) and kamahi (*Weinmannia racemosa)* in areas of dieback. *Pacific Science* **37,** 433-442.

ALLEN, R. B., FITZGERALD, A. E. & EFFORD, M. G. (1997). Long-term changes and seasonal patterns in possum (*Trichosurus vulpecula*) leaf diet, Orongorongo Valley, Wellington, New Zealand. *New Zealand Journal of Ecology* **21,** 181-186.

ANDERSON, R. M. (1978). The regulation of host population growth by parasitic species. *Parasitology* **76,** 119-157.

ANDERSON, R. M. & MAY, R. M. (1978). Regulation and stability of host-parasite population interactions. I. Regulatory processes. *Journal of Animal Ecology* **47,** 219-247.

ANDERSON, R. M. (1995). Evolutionary pressures in the spread and persistence of infectious agents in vertebrate populations. *Parasitology* **111,** S15-31.

ASEA, A., KRAEFT, S. K., KURT-JONES, E. A., STEVENSON, M. A., CHEN, L. B., FINBERG, R. W., KOO, G. C. & CALDERWOOD, S. K. (2000). HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nature Medicine* **6,** 435-442.

ATKINSON, P. E. & WRIGHT, D. E. (1993). The formulation of a national strategy for biological control of possums and bovine Tb. *New Zealand Journal of Zoology* **20,** 325-328.

BASU, S. & SRIVASTAVA, P. K. (2000). Heat shock proteins: the fountainhead of innate and adaptive immune responses. *Cell Stress Chaperones* **5,** 443-451.

BATCHELER, C. L. & COWAN, P. L. (1988). Review of the status of the possum (*Trichosurus vulpecula*) in New Zealand. Ministry of Agriculture and Fisheries, Wellington, New Zealand.

BEALL, J. A. & MITCHELL, G. F. (1986). Identification of a particular antigen from a parasite cDNA library using antibodies affinity purified from selected portions of Western blots. *Jornal of Immunological Methods* **86,** 217-223.

BEERE, H. M. & GREEN, D. R. (2001). Stress management - heat shock protein-70 and the regulation of apoptosis. *Trends in Cell Biology* **11,** 6-10.

BEISSINGER, M. & BUCHNER, J. (1998). How chaperones fold proteins. *Biological Chemistry* **379,** 245-259.

BELOV, K., HARRISON, G. A., MILLER, R. D. & COOPER, D. W. (2001). Characterisation of the kappa light chain of the Brushtail possum (*Trichosurus vulpecula*). *Veterinary Immunology and Immunopathology* **78,** 317-324.

BEVERIDGE, I. & DURETTE-DESSET, M.-C. (1993). Adult and larval stages of *Paraustrostrongylus ratti* (Nematoda: Trichostrongyloidea) from *Rattus fuscipes*. *Transactions of the Royal Society of South Australia* **117,** 27-36.

BEVERIDGE, I. & SPRATT, D. M. (1996). The helminth fauna of Australian marsupials:origins and evolutionary biology. *Advances in Parasitology* **37,** 135-254.

BILLINTON, N. & KNIGHT, A. W. (2001). Seeing the wood through the trees: a review of techniques for distinguishing green fluorescent protein from endogenous autofluorescence. *Analytical Biochemistry* **291,** 175-197.

BLACHERE, N. E., LI, Z., CHANDAWARKAR, R. Y., SUTO, R., JAIKARIA, N. S., BASU, S., UDONO, H. & SRIVASTAVA, P. K. (1997). Heat shock protein-peptide complexes, reconstituted in vitro, elicit peptide-specific cytotoxic T lymphocyte response and tumour immunity. *Journal of Experimental Medicine* **186,** 1315-1322.

BLAKELEY, R. (1993). The Great Lake Pest Summit: anticipated issues and outcomes. *New Zealand Journal of Zoology* **20,** 219-222.

BLAXTER, M. L., DE LEY, P., GAREY, J. R., LIU, L. X., SCHELDEMAN, P., VIERSTRAETE, A., VANFLETEREN, J. R., MACKEY, L. Y., DORRIS, M., FRISSE, L. M., VIDA, J. T. & THOMAS, W. K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* **392,** 71-75.

BLUMENTHAL, T. & STEWARD, K. (1997). RNA processing and gene structure. In *C. elegans II* (ed. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 117-145. Cold Spring Harbour Laboratory Press, Cold Spring Harbour.

BOKHOUT, B. A., VAN GAALEN, C. & VAN DER HEIJDEN, P. J. (1987). A selected water-in-oil emulsion: composition and usefulness as an immunological adjuvant. *Veterinary Immunology and Immunopathology* **2,** 491-500.

BORCHIELLINI, C., BOURY-ESNAULT, N., VACELET, J. & LE PARCO, Y. (1998). Phylogenetic analysis of the Hsp70 sequences reveals the monophyly of Metazoa and specific phylogenetic relationships between animals and fungi. *Molecular Biology and Evolution* **15,** 647-655.

BOWIE, J. & BENNETT, W. (1983). Parasites from possums in Waitahuna forest. *New Zealand Veterinary Journal* **31,** 163.

BOYLE, R., MCLEAN, S., FOLEY, W. J. & DAVIES, N. W. (1999). Comparative metabolism of dietary terpene, p-cymene, in generalist and specialist folivorous marsupials. *Journal of Chemical Ecology* **25,** 2109-2126.

BRENNER, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77,** 71-94.

BRENNER, S. (1988). Forward. In *The nematode Caenorhabditis elegans* (ed. W. B. Wood and The community of *C. elegans* researchers), pp. ix-xiii. Cold Spring Harbour Laboratory, Cold Spring Harbour.

BRITTON, C., REDMOND, D. L., KNOX, D. P., MCKERROW, J. H. & BARRY, J. D. (1999). Identification of promoter elements of parasite nematode genes in transgenic Caenorhabditis elegans. *Molecular and Biochemical Parasitology* **103,** 171-181.

BROWER, V. (2001). Gene therapy revisited: In spite of problems and drawbacks, gene therapy moves forward. *EMBO Reports* **2,** 1064-1065.

BROWN, K., INNES, J. & SHORTEN, R. (1993). Evidence that possums prey on and scavenge bird's eggs, birds and mammals. *Nortornis* **40,** 169-177.

BUDDLE, B. M., WEDLOCK, N. & ALDWELL, F. (1995). Possum Immunology. In *Workshop on Biological Control of Possums*, pp. 19-20. Ministry of Research Science and Technology, Wallaceville, Upper Hutt, New Zealand.

BUKAU, B. & HORWICH, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell* **92,** 351-366.

BURGLIN, T. R., LOBOS, E. & BLAXTER, M. L. (1998). Caenorhabditis elegans as a model for parasitic nematodes. *International Journal for Parasitology* **28,** 395-411.

BURTTON, R. A. (1975). An initial study on the gastrointestinal parasites of the brush-tail opossum, *Trichosurus vulpecula* (Kerr). BSc (Hons) unpublished thesis, Victoria University of Wellington, New Zealand.

CALEY, P. (1998). Broad-scale possum and ferret correlates of macroscopic Mycobacterium bovis infection in feral ferret populations. *New Zealand Veterinary Journal* **46,** 157-162.

CAMERON, P. J., HILL, R. L., BAIN, J. & THOMAS, W. P. (1989). A Review of Biological Control of Invertabrate Pests and Weeds in New Zealand 1874 to 1987, vol. Technical Communication No. 10. CAB International Institute of Biological Control. DSIR Entomology Division.

CAMPBELL, A. (2001). The hatching and growth of *Parastrongyloides trichosuri* larvae incubated at temperatures experienced in the field. Report, unpublished. AgResearch, Wallaceville, Wellington, New Zealand.

CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W. W. & PRASHER, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263,** 802-805.

CHENG, S., FOCKLER, C., M, B. W. & HIGUCHI, R. (1994). Effective amplification of long targets from cloned inserts and human genomic DNA. *Proceedings of the National Academy of Sciences of the USA* **91,** 56695-5699.

CLOUT, M. N. (1982). Determination of age in the brushtail possum using sections from decalcified molar teeth. *New Zealand Journal of Zoology* **9,** 405-408.

CLOUT, M. & ERICKSEN, K. (2000). Anatomy of a disasterous success: the Brushtail possum as an invasive species. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 1-9. Manaaki Whenua Press, Lincoln.

COLEMAN, J., THOMAS, M. D., PRACY, L. T. & HANSEN, Q. (1999). Fluctuation in possum numbers in the Pararaki Valley, Haurangi State Forest Park. In *Science for Conservation*, vol. 128, pp. 16. Department of Conservation, Wellington, New Zealand.

COLEMAN, J. & LIVINGSTONE, P. (2000). Fewer possums less bovine Tb. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 220-231. Manaaki Whenua Press, Lincoln.

COLEMAN, J. & CALEY, P. (2000). Possums as a reservoir of bovine Tb. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 92-104. Manaaki Whenua Press, Lincoln.

COWAN, P. L. & MOEED, A. (1987). Invertabrates in the diet of Brushtail possums, *Trichosurus vulpecula,* in lowland podocarp/broadleaf forest, Orongorongo Valley, Wellington, New Zealand. *New Zealand Journal of Zoology* **14,** 163-177.

COWAN, P. E. (1989). Changes in milk composition during lactation in the common Brushtail possum, Trichosurus vulpecula (Marsupialia: Phalangeridae). *Reproduction, Fertility and Development* **1,** 325-335.

COWAN, P. E. (1990). Brushtail Possum. In *The Handbook of New Zealand Mammals* (ed. C. M. King), pp. 68-98. Oxford University Press, Auckland.

COWAN, P. E. (1993). Effects of intensive trapping on breeding and age structure of brushtail possums *Trichosurus vulpecula,* on Kapiti Island, New Zealand. *New Zealand Journal of Zoology* **20,** 1-11.

COWAN, P. E., BROCKIE, R. E., SMITH, R. N. & HEARFIELD, M. E. (1997). Dispersal of juvenile brushtail possums, *Trichsurus vulpecula*, after a control operation. *Wildlife Research* **24,** 279-288.

COWAN, P., CHILVERS, B. L., EFFORD, M. G. & MCELREA, G. J. (1997 a). Effects of possum browsing on northern rata, Orongorongo Valley,Wellington, New Zealand. *Journal of the Royal Society of New Zealand* **27,** 173-179.

COWAN, P., WALMSLEY, A., KIRK, D., LEE, S. M. & YOUNG, P. (1999). Plant-derived vaccines for possum fertility control. In *Advances in the Biological Control of Possums*, vol. Miscellaneous Series 56 (ed. G. Sutherland), pp. 24-27. The Royal Society of New Zealand, Wallaceville, Upper Hutt, New Zealand.

COWAN, P. E. & CLOUT, M. (2000). Possums on the move: activity patterns, home ranges and dispersal. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 24-34. Manaaki Whenua Press, Lincoln.

COWAN, P. E., CLARK, J. M., HEATH, D. D., STANKIEWICZ, M. & MEERS, J. (2000). Predators, parasites and diseases of possums. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 82-91. Manaaki Whenua Press, Lincoln.

DAVIES, H., MOLLER, H., O'HARA, P., WRIGHT, D. & VELTMAN, C. (2001). Scientific and political challenges to developing effective biolgical control of possums. In *Biological Management of Possums*, pp. 83-98. Ministry of Agriculture and Forestry, Wallaceville, Wellington New Zealand.

DAVIS, R. E., PARRA, A., LOVERDE, P. T., RIBEIRO, E., GLORIOSO, G. & HODGSON, S. (1999). Transient expression of DNA and RNA in parasitic helminths by using particle bombardment. *Proceedings of the National Academy of Sciences of the USA* **96,** 8687-8692.

DEMMER, J., ROSS, I. K., GINGER, M. R., PIOTTE, C. K. & GRIGOR, M. R. (1998). Differential expression of milk protein genes during lactation in the common brushtail possum (Trichosurus vulpecula). *Journal of Molecular Endocrinology* **20,** 37-44.

DIETRICH, P., DRAGATSIS, I., XUAN, S., ZEITLIN, S. & EFSTRATIADIS, A. (2000). Conditional mutagenesis in mice with heat shock promoter-driven cre transgenes. *Mammalian Genome* **11,** 196-205.

DOOLIN, E. E., WEDLOCK, D. N., BUDDLE, B. M. & SKINNER, M. A. Mucosal immunity in the brushtail possum (*Trichosurus vulpecula*): Detection of antibody in serum and at female reproductive sites after intranasal immunisation. *Immunology and Cell Biology* **in press**.

DOOLIN, E. E., MIDWINTER, R. G., MCCARTHY, A. R. & BUDDLE, B. M. (2001). Purification of secretory immunoglobulin A from milk of the brushtail possum (Trichosurus vulpecula). *New Zealand Veterinary Journal* **49,** 181-186.

DUCKWORTH, J., HARRIS, M., MATE, K. E., MCCARTNEY, C., BUIST, J., SCOBIE, S., JONES, D. & JONES, S. (1999). Development of brushtail possum immunocontraception: targetting sperm and egg antigens. In *Advances in the Biological Control of Possums*, vol. Miscellaneous Series 56 (ed. G.

Sutherland), pp. 72-76. The Royal Society of New Zealand, Wallaceville, Upper Hutt, New Zealand.

EASON, C. T., FRAMPTON, C. M., HENDERSON, R., THOMAS, M. D. & MORGAN, D. R. (1993). Sodium monofluoroacetate and alternative toxins for possum control. *New Zealand Journal of Zoology* **20,** 329-334.

EASTON, D. P., KANEKO, Y. & SUBJECK, J. R. (2000). The Hsp110 and Grp 170 stress proteins: newly recognized relatives ef the Hsp70s. *Cell Stress and Chaperones* **5,** 276-290.

ECKERY, D. C., JUENGEL, J. L., BAUER, E., HEATH, D. A., LAWRENCE, S. B., LUN, S., MCLEOD, B. J., MOORE, L. G., THOMPSON, E. G, THOMSON, B P., WESTERN, A., WHALE, L. & MCNATTY, K. P. (2001). Disabling reproductive function in possums:current strategies, research findings and future directions. In *Biological Management of Possums*, pp. 73-82. Ministry of Agriculture and Forestry, Wallaceville, Wellington New Zealand.

EFFORD, M. G. (2000). Possum density, population structure and dynamics. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 47-61. Manaaki Whenua Press, Lincoln.

EKDAHL, M. O., SMITH, B. L. & MOREY, B. L. (1970). Tuberculosis in some wild and feral animals in New Zealand. *New Zealand Veterinary Journal* **18,** 44-45.

EMERY, D. L. (1996). Vaccination against worm parasites of animals. *Veterinary Parasitology* **64,** 31-45.

FEIGE, U. & POLLA, B. S. (1994). Heat shock proteins: the hsp70 family. *Experentia* **50,** 979-986.

FENNER, F. & FANTINI, B. (1999). Biological control of vertabrate pests: the history of myxomatosis, an experiment in evolution., pp. 339. CABI Publishing, Oxford.

FIRE, A. (1986). Integrative transformation of *Caenorhabditis elegans*. *The EMBO Journal* **5,** 2673-2680.

FIRE, A., HARRISON, S. W. & DIXON, D. (1990). A modular set of lacZ fusion vectors for studying gene expression in Caenorhabditis elegans. *Gene* **93,** 189-198.

FIRE, A. (1992). Histochemical techniques for locating Escherichia coli beta-galactosidase activity in transgenic organisms. *Genetic Analysis, Techniques and Applications* **9,** 151-158.

FISCHER, B., SUMNER, I. & GOODENOUGH, P. (1992). Isolation and renaturation of bio-active proteins expressed in Escherichia coli as inclusion bodies. *Arzneimittelforschung* **42,** 1512-1515.

FITZGERALD, A. E. (1984). Diet of the opossum (*Trichosurus vulpecula*) in three Tasmanian forest types and its relevance to the diet of possums in New Zealand forests. In *Possums and Gliders* (ed. A. P. Smith and I. D. Hume), pp. 137-143.

Australian Mammal Society, Chipping Norton, NSW.

FITZGERALD, A. E. (1984 a). Diet overlap between kokako and the common brushtail possum in central North Island, New Zealand. In *Possums and Gliders* (ed. A. P. Smith and I. D. Hume), pp. 569-573. Australian Mammal Society, Chipping Norton, NSW.

FITZGERALD, B. M. & GIBB, J. A. (2001). Introduced mammals in a New Zealand forest: long-term research in the Orongorongo Valley. *Biological Conservation* **99,** 97-108.

FLAHERTY, K. M., DELUCA-FLAHERTY, C. & MCKAY, D. B. (1990). Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature* **346,** 623-628.

FLANAGAN, J., HEATH, D. D., STANKIEWICZ, M. & COWAN, P. Comparison of necropsy and serology for epidemiological studies of *Parastrongyloides trichosuri* in the brushtail possum (*Trichosurus vulpecula*) in New Zealand. Unpublished.

FLETCHER, T. & SELWOOD, L. (2000). Possum reproduction and development. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 62-81. Manaaki Whenua Press, Lincoln.

FOLTZ, K. R., PARTIN, J. S. & LENNARZ, W. J. (1993). Sea urchin egg receptor for sperm: sequence similarity of binding domain and hsp70. *Science* **259,** 1421-1425.

FREELAND, W. J. & WINTER, J. W. (1975). Evolutionary consequences of eating: *Trichosurus vulpecula* (Marsupialia) and the genus *Eucalyptus*. *Journal of Chemical Ecology* **1,** 439-455.

FREEMAN, B. C., MYERS, M. P., SCHUMACHER, R. & MORIMOTO, R. I. (1995). Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. *The EMBO Journal* **14,** 2281-2292.

FRYDMAN, J. (2001). Folding of newly translated proteins in vivo: The role of molecular chaperones. *Annual Review of Biochemistry* **70,** 603-647.

GAUGLER, R., WILSON, M. & SHEARER, P. (1997). Field release and environmental fate of a transgenic entomopathogenic nematode. *Biological Control* **9,** 75-80.

GEMMEL, R. T., CEPON, G. & BARNES, A. (1986). Weekly variations in body weight and plasma testosterone concentrations in the captive male possum *Trichosurus vulpecula*. *General and Comparative Endocrinology* **62**, 1-7.

GRAHAM-ROWE, D. (2000). Possums on the pill - contraceptive carrots will stop the spread of marauding marsupials. *New Scientist* **165,** 18.

GRANT, W. N. (1992). Transformation of *Caenorhabditis elegans* with genes from parasitic nematodes. *Parasitology Today* **8,** 344-346.

GRANT, W. N. & VINEY, M. E. (2001). Post-genomic nematode parasitology. *International Journal for Parasitology* **31,** 879-888.

GREEN, W. Q. & COLEMAN, J. D. (1984). Response of a brushtailed possum population to intensive trapping. *New Zealand Journal of Zoology* **11,** 319-328.

GREEN, W. Q. (1984). A review of ecological studies relevant to management of the common brushtail possum. In *Possums and Gliders* (ed. A. P. Smith and I. D. Hume), pp. 483-499. Australian Mammal Society, Chipping Norton NSW.

GRENFELL, B. & GULLAND, F. M. (1995). Introduction: Ecological impact of parasitism on wildlife host populations. *Parasitology* **111,** S1-S14.

GROSSHANS, H. (2000). Gene therapy - when a simple concept meets a complex reality: Review on gene therapy. *Functional and Integrative Genomics* **1,** 142-145.

GRUENBERG, A. & BISSET, S. (1997). Parastrongyloides trichosuri as a potential vector for genes expressing proteins which could interfere with reproductive success, growth or longevity of brushtailed possums. In *Biological Control of Possums*, vol. Miscellaneous Series 45 (ed. R. Lynch), pp. 10-12. The Royal Society of New Zealand, Wallaceville, Upper Hutt, New Zealand.

GRUENBERG, A., BISSET, S., RALSTON, M., HEATH, D. D. & SHOEMAKER, C. (1999). Towards a transgenic possum parasite. In *Advances in the biological control of possums*, vol. Miscellaneous Series 56 (ed. G. Sutherland), pp. 10. The Royal Society of New Zealand, Wallaceville, Upper Hutt, New Zealand.

GUNTHER, E. & WALTER, L. (1994). Genetic aspects of the hsp70 multigene family in vertabrates. *Experentia* **50,** 987-1001.

GUPTA, R. S. & GOLDING, G. B. (1993). Evolution of HSP70 gene and its implications regarding relationships between archaebacteria, eubacteria, and eukaryotes. *Journal of Molecular Evolution* **37,** 573-582.

GUZMAN, L.-M., BELIN, D., CARSON, M. & BECKWITH, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinoseP_{BAD} promoter. *Journal of Bacteriology* **177,** 4121-4130.

HAMES, B. D. & RICKWOOD, D. (1981). *Gel electrophoresis of proteins: a practical approach.* IRL Press, Oxford.

HAN, W. J. & CHRISTEN, P. (2001). Mutations in the interdomain linker region of DnaK abolish the chaperone action of the DnaK/DnaJ/GrpE system. *FEBS Letters* **497,** 55-58.

HARRIS, M. S., CUI, X., JONES, S., MCCARTNEY, C. & O'RAND, M. G. (2001). Identification of new antigen targets and the molecular dissection of antigenicity for possum immunocontraception. In *Biological Management of Possums*, pp. 20-23. Ministry of Agriculture and Forestry, Wallaceville, Wellington New Zealand.

HARRISON, G. A. & WEDLOCK, D. N. (2000). Marsupial cytokines - Structure, function and evolution. *Developmental and Comparative Immunology* **24,** 473-484.

HARTL, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature* **381,** 571- 580.

HASHMI, S., HASHMI, G. & GAUGLER, R. (1995 a). Genetic transformation of an entomopathogenic nematode by microinjection. *Journal of Invertebrate Pathology* **66,** 293-296.

HASHMI, S., LING, P., HASHMI, G., REED, M., GAUGLER, R. & TRIMMER, W. (1995 b). Genetic transformation of nematodes using arrays of micromechanical piercing structures. *Biotechniques* **19,** 766-770.

HASHMI, S., HASHMI, G., GLAZER, I. & GAUGLER, R. (1998). Thermal response of Heterorhabditis bacteriophora transformed with the Caenorhabditis elegans hsp70 encoding gene. *Journal of Experimental Zoology* **281,** 164-170.

HASHMI, S., TAWE, W. & LUSTIGMAN, S. (2001). Caenorhabditis elegans and the study of gene function in parasites. *Trends in Parasitology* **17,** 387-393.

HEATH, D. D. & LAWRENCE, S. B. (1996). Antigenic polypeptides of Echinococcus granulosus oncospheres and definition of protective molecules. *Parasite Immunology* **18,** 347-357.

HEATH, D. D., COWAN, P., STANKIEWICZ, M., CLARK, J. M., HORNER, G., TEMPERO, J., JOWETT, G., FLANAGAN, J., SHUBBER, A., STREET, L., MCELREA, G. J., CHILVERS, B. L., NEWTON-HOWES, J., JOWETT, J. & MORRISON, L. (1997). Possum biological control - parasites and bacteria. In *Biological Control of Possums* (ed. R. Lynch), pp. 13-19. The Royal Society of New Zealand Miscellaneous Series 45, Wallaceville, Upper Hutt, New Zealand.

HEATH, D. D., STANKIEWICZ, M., JOWETT, G., FLANAGAN, J. & COWAN, P. (1999). Immunological studies of *Parastrongyloides trichosuri* (Nematoda) in brushtail possums, *Trichosurus vulpecula*. *Acta Parasitologica* **44,** 131-136.

HEMSLEY, S. W., CANFIELD, P. J. & HUSBAND, A. J. (1996). The distribution of organised lymphoid tissue in the alimentary tracts of koalas (Phascolarctos cinereus) and possums (Trichosurus vulpecula and Pseudocheirus peregrinus). *Journal of Anatomy* **188,** 269-278.

HOCHACHKA, W. M. & DHONDT, A. A. (2000). Density-dependent decline of host abundance resulting from a new infectious disease. *Proceedings of the National Academy of Sciences of the USA* **97,** 5303-5306.

HOPE , R., COOPER, S. & WAINWRIGHT, B. (1990). Globin macromolecular sequences in marsupials and monotremes. *Australian Journal of Zoology* **37,** 289-313.

HOPE, I. A. (1991). 'Promoter trapping' in Caenorhabditis elegans. *Development* **113,** 399-408.

HUDSON, P. J., DOBSON, A. P. & NEWBORN, D. (1998). Prevention of population cycles by parasite removal. *Science* **282,** 2256-2258.

HUMPHREYS, W. F., BRADLEY, A. J., HOW, R. A. & L, B. J. (1984). Indices of condition of phalanger population: a review. In *Possums and Gliders* (ed. A. Smith and I. Hume), pp59-77. Australian Mammal Society, Chipping Norton, NSW.

INNES, J. (1994). The impacts of possums on native fauna. In *Possums as Conservation Pests* (ed. C. F. J. O'Donnell), pp. 11-16. Department of Conservation, Christchurch, New Zealand.

JACKSON, R., COOKE, M. M., COLEMAN, J. D., MORRIS, R. S., DE LISLE, G. W. & YATES, G. F. (1995). Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichosurus vulpecula*): III. Routes of infection and excretion. *New Zealand Veterinary Journal* **43,** 322-3327.

JACKSON, R. & MORRIS, R. S. (1996). A study of the topography of the lymphatic system of the Australian brushtail possum (Trichosurus vulpecula). *Journal of Anatomy* **188,** 603-609.

JACKSTADT, P., WILM, T. P., ZAHNER, H. & HOBOM, G. (1999). Transformation of nematodes via ballistic DNA transfer. *Molecular and Biochemical Parasitology* **103,** 261-266.

JAENICKE, R. (1998). Protein self-organization *in vitro* and *in vivo*: partioning between physical biochemistry and cell biology. *Biological Chemistry* **379,** 237-243.

JAN, E., YOON, J. W., WALTERHOUSE, D., IANNACCONE, P. & GOODWIN, E. B. (1997). Conservation of the *C. elegans* tra-2 3'UTR translational control. *The EMBO Journal* **16,** 6301-13.

JANSON, K. W. (1994). Prioritisation and optimisation. In *Possums as Conservation Pests* (ed. C. F. J. O'Donnell), pp. 25-28. Department of Conservation, Christchurch, New Zealand.

JARRETT, W. F. H., JENNINGS, F. W., MARTIN, B., MCINTYRE, W. I. M., MULLIGAN, W., SHARP, N. C. C. & URQUHART, G. M. (1958). A field trial of a parasitic bronchitis vaccine. *Veterinary Record* **70,** 451-454.

JAYASENA, S. M., CHANDRASEKHARAN, N. V. & KARUNANAYAKE, E. H. (1999). Molecular characterisation of a hsp70 gene from the filarial parasite *Setaria digitata*. *International Journal for Parasitology* **29,** 581-591.

JOHNSON, K. S., HARRISON, G. B., LIGHTOWLERS, M. W., O'HOY, K. L., COUGLE, W. G., DEMPSTER, R. P., LAWRENCE, S. B., VINTON, J. G., HEATH, D. D. & RICKARD, M. D. (1989). Vaccination against ovine cysticercosis using a defined recombinant antigen. *Nature* **338,** 585-587.

JOLLY, S. E. (1993). Biological control of possums. *New Zealand Journal of Zoology* **20,** 335-339.

JONES, D., STRINGHAM, E. G., BABICH, S. L. & CANDIDO, E. P. (1996). Transgenic strains of the nematode *C. elegans* in biomonitoring and toxicology: effects of captan and related compounds on the stress response. *Toxicology* **109,** 119-127.

JUNKERSDORF, B., BAUER, H. & GUTZEIT, H. O. (2000). Electromagnetic fields enhance the stress response at elevated temperatures of the nematode *C. elegans*. *Bioelectromagnetics* **21,** 100-106.

KARLIN, S. & BROCCHIERI, L. (1998). Heat shock protein 70 family: multiple sequence comparisons, function, and evolution. *Journal of Molecular Evolution* **47,** 565-577.

KELTON, S. (1994). Control technology application - what currently limits our effectiveness. In *Possums as Conservation Pests* (ed. C. F. J. O'Donnell), pp. 57-62. Department of Conservation, Christchurch, New Zealand.

KERLE, J. A. (1984). Variations in the ecology of *Trichosurus:* its adaptative significance. In *Possums and Gliders* (ed. A. P. Smith and I. D. Hume), pp. 115-128. Australian Mammal Society, Sydney.

KHALIL, L. F. (1970). *Bertiella trichosuri* n. sp. from the bush-tail opossum *Trichosurus vulpecula* (Kerr) from New Zealand. *Zoologischer Anzeiger* **185,** 442-450.

KIMBLE, J., HODGKIN, J., SMITH, T. & SMITH, J. (1982). Suppression of an amber mutation by microinjection of suppressor tRNA in C. elegans. *Nature* **299,** 456-458.

KING, D. R., OLIVER, A. J. & MEAD, R. J. (1978). The adaptation of some Western Australian mammals to food plants containing fluoroacetate. *Australian Journal of Zoology* **26,** 699-712.

KNOX, D. P. (2000). Development of vaccines against gastrointestinal nematodes. *Parasitology* **120,** S43-61.

KO, R. C. & FAN, L. (1996). Heat shock response of *Trichinella spiralis* and *T. pseudospiralis*. *Parasitology* **112,** 89-95.

KRAUSE, M., HARRISON, S. W., XU, S. Q., CHEN, L. & FIRE, A. (1994). Elements regulating cell- and stage-specific expression of the C. elegans MyoD family homolog hlh-1. *Developmental Biology* **166,** 133-148.

KRAUSE, S., SOMMER, A., FISCHER, P., BROPHY, P. M., WALTER, R. D. & LIEBAU, E. (2001). Gene structure of the extracellular glutathione S-transferase from Onchocerca volvulus and its overexpression and promoter analysis in transgenic Caenorhabditis elegans. *Molecular and Biochemical Parasitology* **117,** 145-154.

KWA, M. S., VEENSTRA, J. G., VAN DIJK, M. & ROOS, M. H. (1995). Beta-tubulin genes from the parasitic nematode Haemonchus contortus modulate drug resistance in Caenorhabditis elegans. *Journal of Molecular Biology* **246,** 500-510.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277,** 680-685.

LAWLER, I. R., FOLEY, W. J., ESCHLER, B. M., PASS, D. M. & HANDASYDE, K. (1998). Intraspecific variation in Eucalyptus secondary metabolites determines food intake by folivorous marsupials. *Oecologia* **116,** 160-169.

LILIE, H., SCHWARZ, E. & RUDOLPH, R. (1998). Advances in refolding of proteins produced in E. coli. *Current Opinion in Biotechnology* **9,** 497-501.

LIN, M., ZHANG, X., WADE, M., HARRIS, M., HARMAN, A. & RODGER, J. C. (1997). Sperm acrosome formation in the tammar wallaby and the brushtail possum. In *Biological Control of Possums*, vol. Miscellaneous Series 45 (ed. R. Lynch), pp. 73-75. The Royal Society of New Zealand, Wallaceville, Upper Hutt, New Zealand.

LIS, J. (1998). Promoter-associated pausing in promoter architecture and postinitiation transcriptional regulation. *Cold Spring Harb Symp Quant Biol* **63,** 347-56.

LINDQUIST, S. & CRAIG, E. A. (1988). The heat-shock proteins. *Annual Review of Genetics* **22,** 631-677.

LINK, C. D., CYPSER, J. R., JOHNSON, C. J. & JOHNSON, T. E. (1999). Direct observation of stress response in Caenorhabditis elegans using a reporter transgene. *Cell Stress Chaperones* **4,** 235-242.

LIVINGSTONE, P. (1994). Infrared camera trial to identify possum and other feral/wild animal density/location. In *Possums as Conservation Pests* (ed. C. F. J. O'Donnell), pp. 53-56. Department of Conservation, Christchurch, New Zealand.

LOK, J. B. & MASSEY, H. C. (2002). Transgene expression in Strongyloides stercoralis following gonadal microinjection of DNA constructs. *Molecular and Biochemical Parasitology* **119,** 279-84.

LUDERS, J., DEMAND, J. & HOHFELD, J. (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *Journal of Biological Chemistry* **275,** 4613-4617.

LUGTON, I. W., WILSON, P. R., MORRIS, R. S. & NUGENT, G. (1988). Epidemiology and pathogenesis of *Mcobacterium bovis* infection of red deer (*Cervus elaphus*) in New Zealand. *New Zealand Veterinary Journal* **46,** 147-156.

MACKERRAS, M. J. (1959). *Strongyloides* and *parastrongyloides* (Nematoda: Rhabdiasoidea) in Australian marsupials. *Australian Journal of Zoology* **7,** 87-104.

MARONEY, P. A., DENKER, J. A., DARZYNKIEWICZ, E., LANEVE, R. & NILSEN, T. W. (1995). Most mRNAs in the nematode Ascaris lumbricoides are trans-spliced: a role for spliced leader addition in translational efficiency. *Rna* **1,** 714-723.

MARSHALL, D. (1993). Present government policy on pest control. *New Zealand Journal of Zoology* **20,** 321-323.

MARSTON, F. A. O. (1986). The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Journal of Biochemistry* **240,** 1-12.

MARTINEZ, J., PEREZ-SERRANO, J., BERNADINA, W. E. & RODRIGUEZ-CAABEIRO, F. (2001). HSP60, HSP70 and HSP90 from Trichinella spiralis as targets of humoral immune response in rats. *Parasitology Research* **87,** 453-8.

MATZINGER, P. (1994). Tolerance, danger, and the extended family. *Annual Review of Immunology* **12,** 991-1045.

MAWSON, P. M. (1973). Amidostomatinae (Nematoda: Trichostrongyloidea) from Australian marsupials and monotremes. *Transactions of the Royal Society of South Australia* **97,** 257-279.

MAY, R. M. & ANDERSON, R. M. (1978). Regulation and stability of host-parasite interactions. II Destabilizing processes. *Journal of Animal Ecology* **47,** 249-267.

MAYER, M. P. & BUKAU, B. (1998). Hsp70 chaperone systems: diversity of cellular functions and mechanism of action. *Biological Chemistry* **379,** 261-263.

MCGHEE, J. D. & KRAUSE, M. W. (1997). Transcriptional Factors and Transcriptional Regulation. In *C. Elegans II* (ed. D. L. Riddle, T. Blumenthal, B. Meyer and J. R. Priess), pp. 169-184. Cold Spring Harbour Laboratory Press.

MCINTOSH, C. G., CRAWFORD, J. L., THOMSON, E. G., MCLEOD, B. J., GILL, J. M. & O'KEEFE, J. S. (1995). A newly discovered disease of the brushtail possum: wobbly possum syndrome. *New Zealand Veterinary Journal* **43,** 126.

MELLO, C. C., KRAMER, J. M., STINCHCOMB, D. & AMBROS, B. (1991). Efficient gene transfer in C. elegans: Extrachromosomal maintenance and integration of transforming sequences. *The EMBO Journal* **10,** 3959-3970.

MILLER, R. D. & BELOV, K. (2000). Immunoglobulin genetics of marsupials. *Developmental and Comparative Immunology* **24,** 485-490.

MOLINIA, F. C., GLAZIER, A. M., MYERS, J. V., SIDHU, K. S., MATE, K. E., BERG, D. K., SMITH, J. F., COWAN, P. E. & RODGER, J. C. (2001). In vitro and in vivo models of key reproductive events as tools for possum biological control. In *Biological Management of Possums*, pp. 1-5. Ministry of Agriculture and Forestry, Wallaceville, Upper Hutt, New Zealand.

MORGAN, D. O. (1928). *Parastrongyloides winchesi* gen. et sp. nov. A remarkable new nematode parasite of the mole and the shrew. *Journal of Helminthology* **6,** 79-86.

MORGAN, D. R. (1990). Behavioural responses of brushtailed possums (*Trichosurus vulpecula*) to baits used in pest control. *Australian Wildlife Research* **17,** 610-613.

MORGAN, D. R. (2000). Caught in the Headlights: New Zealander's Reflections on Possums, Control Options and Genetic Engineering, pp. 13-15. The Parliamentary Commissioner for the Environment, Wellington, New Zealand.

MORGAN, D. R. & HICKLING, G. J. (2000). Techniques used for poisoning possums. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 143-153. Manaaki Whenua Press, Lincoln.

MORIMOTO, R. I. (1993). Cells in stress: transcriptional activation of heat shock genes. *Science* **259,** 1409-1410.

MORIMOTO, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Development* **12,** 3788-3796.

MOSELEY, P. (2000). Stress proteins and the immune response. *Immunopharmacology* **48,** 299-302.

MULTHOFF, G., BOTZLER, C. & ISSELS, R. (1998). The role of heat shock proteins in the stimulation of an immune response. *Biological Chemistry* **379,** 295-300.

MUTWAKIL, M. H., READER, J. P., HOLDICH, D. M., SMITHURST, P. R., CANDIDO, E. P. M., JONES, D., STRINGHAM, E. G. & DE POMERAI, D. I. (1997). Use of stress-inducible transgenic nematodes as biomarkers of heavy metal pollution in water samples from an english river system. *Archives of Environmental Contamination and Toxicology* **32,** 146-153.

NEWTON, S. E. & MUNN, E. A. (1999). The development of vaccines against gastrointestinal nematode parasites, particularly Haemonchus contortus. *Parasitology Today* **15,** 116-122.

NILSEN, T. W. (1993). Trans-splicing of nematode premessenger RNA. *Annual Review of Microbiology* **47,** 413-440.

NOWELL, M. A., DEPOMERAI, D. I. & PRITCHARD, L. I. (1999). Caenorhabditis elegans as a biomonitor for immunological stress in nematodes. *Parasite Immunology* **21,** 495-505.

NUGENT, G. (1994). Effects of possums on the native flora. In *Possums as Conservation Pests* (ed. C. F. J. O'Donnell), pp. 5-10. Department of Conservation, Christchurch, New Zealand.

NUGENT, G., SWEETAPPLE, P., COLEMAN, J. & SUISTED, P. (2000). Possum feeding patterns:dietary patterns of a reluctant folivore. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 10-23. Manaaki Whenua Press, Lincoln.

OBENDORF, D., SPRATT, D. M., BEVERIDGE, I., PRESIDENTE, P. J. A. & COMAN, B. (1997). Parasites and diseases in Australian brushtail possums, *Trichosurus* spp. In *Biological Control of Possums*, vol. Miscellaneous Series 45 (ed. R. Lynch), pp. 6-9. The Royal Society of New Zealand, Wallaceville, Upper Hutt, New

Zealand.

O'KEEFE, J. S., STANISLAWEK, W. L. & HEATH, D. D. (1997). Pathological studies of wobbly possum disease in New Zealand brushtail possums (*Trichosurus vulpecula*). *Veterinary Record* **141,** 226-229.

OLD, J. M. & DEANE, E. M. (2000). Development of the immune system and immunological protection in marsupial pouch young. *Developmental and Comparative Immunology* **24,** 445-454.

OPPERMAN, C. H. & BIRD, D. M. (1998). The soybean cyst nematode, Heterodera glycines: a genetic model system for the study of plant-parasitic nematodes. *Current Opinion in Plant Biology* **1,** 342-346.

PARLIAMENTARY COMMISSIONER FOR THE ENVIRONMENT. (1994). Possum Management in New Zealand, pp. 196. Office of the Parliamentary Commissioner for the Environment, Wellington, New Zealand.

PARLIAMENTARY COMMISSIONER FOR THE ENVIRONMENT. (2000). Caught in the Headlights: New Zealander's Reflections on Possums, Control Options and Genetic Engineering., pp84 Office of the Parliamentary Commissioner for the Environment, Wellington, New Zealand.

PASS, D. M., FOLEY, W. J. & BOWDEN, B. (1998). Vertebrate herbivory on Eucalyptus - Identification of specific feeding deterrents for common ringtail possums (Pseudocheirus peregrinus) by bioassay-guided fractionation of Eucalyptus ovata foliage. *Journal of Chemical Ecology* **24,** 1513-1527.

PATERSON, B. M. & MORRIS, R. S. (1995). Interactions between beef cattle and simulated tuberculous possums on pasture. *New Zealand Veterinary Journal* **43,** 289-293.

PAYTON, I. (2000). Damage to native forests. In *The Brushtail Possum: Biology, Impact and Management of an Introduced Marsupial* (ed. T. L. Montague), pp. 111-125. Manaaki Whenua Press, Lincoln.

PELHAM, H. R. B. (1982). A regulatory upstream promoter in the *Drosophila* hsp70 heat shock gene. *Cell* **30,** 517-528.

PELHAM, H. R. B. (1985). Activation of heat-shock genes in eukaryotes. *Trends in Genetics* **1,** 31-35.

POLLA, B. S. (1991). Heat shock proteins in host-parasite interactions. *Immunology Today* **12,** A38-41.

PONOMAREV, E. D., TARASENKO, T. N. & SAPOZHNIKOV, A. M. (2000). Splenic cytotoxic cells recognize surface HSP70 on culture-adapted EL-4 mouse lymphoma cells. *Immunology Letters* **74,** 133-139.

PRACY, L. T. & KEAN, R. I. (1968). Tapeworms found in opossums. *New Zealand Journal of Agriculture* **117,** 21-22.

PRACY, L. T. (1974). *Introduction and liberation of the opossum (Trichosurus vulpecula) into New Zealand.*, 2nd edition. New Zealand Forest Service Information Series No. 45.

PRESIDENTE, P. J. A., BARNETT, J. L., HOW, R. A. & HUMPHREYS, W. F. (1982). Effects of habitat, host sex and age on the parasites of *Trichosurus caninus* (Marsupialia: Phalangeridae) in northern New South Wales. *Australian Journal of Zoology* **30,** 33-47.

PRESIDENTE, P. J. A. (1984). Parasites and diseases of brushtail possums (*Trichosurus spp*): occurrence and significance. In *Possums and Gliders* (ed. A. P. Smith and I. D. Hume), pp. 438-499. Australian Mammal Society, Sydney.

QIN, L., SMANT, G., STOKKERMANS, J., BAKKER, J., SCHOTS, A. & HELDER, J. (1998). Cloning of a trans-spliced glyceraldehyde-3-phosphate-dehydrogenase gene from the potato cyst nematode Globodera rostochiensis and expression of its putative promoter region in Caenorhabditis elegans. *Molecular and Biochemical Parasitology* **96,** 59-67.

QUIJADA, L., SOTO, M., ALONSO, C. & REQUENA, J. M. (2000). Identification of a putative regulatory element in the 3'-untranslated region that controls expression of HSP70 in Leishmania infantum. *Molecular and Biochemical Parasitology* **110,** 79-91.

RALSTON, M. Length of time that L3 larvae of *Parastrongyloides trichosuri* remain infective in possum faeces in the bush and in the laboratory. Report in progress

RALSTON, M. Species specificity of *Parastrongyloides trichosuri*. Report in progress.

RALSTON, M., COWAN, P. & HEATH, D. D. (1998). A field trial on the effects of parasites on the possum fecundity. In *New Zealand Society for Parasitology. Annual Meeting No. 27*, Lincoln University.

RALSTON, M., COWAN, P. & HEATH, D. D. (1999). Dynamics of the spread of *Parastrongyloides trichosuri* amongst brushtail possums (*Trichosuri vulpecula*) in the South Island of New Zealand. In *Advances in the biological control of possums*, vol. Miscellaneous Series 56 (ed. G. Sutherland), pp. 4-6. Royal Society of New Zealand, Wallaceville.

RALSTON, M., COWAN, P. & HEATH, D. D. (2001). Measuring the spread of the candidate possum biocontrol vector: *Parastrongyloides trichosuri*. In *Biological Management of Possums*, pp. 56-61. Ministry of Agriculture and Forestry, Wallaceville, Wellington New Zealand.

RALSTON, M., STANKIEWICZ, M. & HEATH, D. D. (2001 a). Anthelmintics for the control of nematode infections in the brushtail possum (*Trichosurus vulpecula*). *New Zealand Veterinary Journal* **49,** 73-77.

RAMADASS, P. & MORIARTY, K. M. (1982). Immunoglobulins of the Australian brushtail possum, *Trichosurus vulpecula*. *Developmental and Comparative Immunology* **6,** 549-556.

RANFORD, J. C., COATES, A. R. M. & HENDERSON, B. (2000). Chaperonins are cell-signalling proteins: the unfolding biology of molecular chaperones. Expert Reviews in Molecular Medicine. URL: http:// www.ermm.cbcu.cam.ac.uk /00002015h. htm

REDMOND, D. L., CLUCAS, C., JOHNSTONE, I. L. & KNOX, D. P. (2001). Expression of Haemonchus contortus pepsinogen in Caenorhabditis elegans. *Molecular and Biochemical Parasitology* **112,** 125-131.

RICE, M. & WILKS, C. R. (1996). Virus and virus-like particles observed in the intestinal contents of the possum, *Trichosurus vulpecula. Archives of Virology* **141,** 945- RICKARD, M. D., HARRISON, G. B., HEATH, D. D. & LIGHTOWLERS, M. W. (1995). Taenia ovis recombinant vaccine-'quo vadit'. *Parasitology* **110,** S5-9.

RODGER, J. C., SIDHU, K. S., MATE, K. E. & HARMAN, A. (2001). Recent developments in IVF and oviduct biology: new experimental tools and biocontrol targets. In *Biological Management of Possums*, pp. 12-13. Ministry of Agriculture and Forestry, Wallaceville, Wellington, New Zealand.

ROSE, A. B., PEKELHARING, C. L. & PLATT, K. H. (1992). Magnitude of canopy dieback and implications for conservation of Southland rata-kamahi (*Metrosideros umbellata-Weinmannia racemosa*) forests, central Westland, New Zealand. *New Zealand Journal of Ecology* **16,** 23-32.

ROTHSTEIN, N. & RAJAN, T. V. (1991). Characterization of an hsp70 gene from the human filarial parasite, Brugia malayi (Nematoda). *Molecular and Biochemical Parasitology* **49,** 229-237.

ROYAL COMMISSION ON GENETIC MODIFICATION. (2001). Report of the Royal Commission on Genetic Modification, Wellington, New Zealand.

RUDIGER, S., BUCHBERGER, A. & BUKAU, B. (1997). Interaction of Hsp70 chaperones with substrates. *Nature Structural Biology* **4,** 342-349.

RUEF, B. J., WARD, T. J., OXNER, C. R., CONLEY, P. G., BROWN, W. C. & RICE-FICHT, A. C. (2000). Phylogenetic analysis with newly characterized Babesia bovis hsp70 and hsp90 provides strong support for paraphyly within the piroplasms. *Molecular and Biochemical Parasitology* **109,** 67-72.

SAMISH, M. & GLAZER, I. (2001). Entomopathogenic nematodes for the biocontrol of ticks. *Trends in Parasitology* **17,** 368-371.

SCHRODA, M., BLOCKER, D. & BECK, C. F. (2000). The HSP70A promoter as a tool for the improved expression of transgenes in Chlamydomonas. *The Plant Journal* **21,** 121-31.

SCIEGLINSKA, D., WIADMACRLAK, W., KONOPKA, W., POUTANEN, M., RAHMAN, N., HUHTANIEMI, I. & KRAWCZYK, Z. (2001). Structure of the 5' region of the Hst70 gene transcription unit: presence of an intron and multiple transcription initiation sites. *The Biochemical Journal* **359,** 129-137.

SCOTT, M. E. (1987). Regulation of mouse colony abundance by Heligmosomoides polygyrus. *Parasitology* **95,** 111-124.

SCOTT, M. E. (1990). An experimental and theoretical study of the dynamics of a mouse-nematode (Heligmosomoides polygyrus) interaction. *Parasitology* **101,** 75-92.

SEED, B., PARKER, R. C. & DAVIDSON, N. (1982). Representation of DNA sequences in recombinant DNA libraries prepared by restriction enzyme partial digestion. *Gene* **19,** 201-209.

SELKIRK, M. E., RUTHERFORD, P. J., DENHAM, D. A., PARTONO, F. & MAIZELS, R. M. (1987). Cloned antigen genes of Brugia filarial parasites. *Biochemical Society Symposium* **53,** 91-102.

SELKIRK, M. E., DENHAM, D. A., PARTONO, F. & MAIZELS, R. M. (1989). Heat shock cognate 70 is a prominent immunogen in Brugian filariasis. *Journal of Immunology* **143,** 299-308.

SHEVCHENKO, A., WILM, M., VORM, O. & MANN, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analytical Chemistry* **68,** 850-858.

SINGLETON, G. R. & CHAMBERS, L. K. (1996). A manipulative field experiment to examine the effect of Capillaria hepatica (Nematoda) on wild mouse populations in southern Australia. *International Journal for Parasitology* **26,** 383-398.

SKINNER, S. J. M. (2000). Maintenance of *Parastrongyloides trichosuri* in culture both before and after micro injection. Unpublished.

SKINNER, S. J. M., NEWTON-HOWES, J., RALSTON, M., HEATH, D. D. & SHOEMAKER, C. (2001). Potential transgenic nematode parasites for continual production of bioactive agents for possum control. In *Biological Control of Possums*, vol. Miscellaneous Series. Royal Society of New Zealand, Wallaceville, Upper Hutt, New Zealand.

SMITH, A. P. & HUME, I. D. (1984). Possums and Gliders. Australian Mammal Society, Chipping Norton NSW.

SNUTCH, T. P., HESCHL, M. F. & BAILLIE, D. L. (1988). The *Caenorhabditis elegans hsp70* gene family: a molecular genetic characterization. *Gene* **64,** 241-255.

SONDERMANN, H., SCHEUFLER, C., SCHNEIDER, C., HOHFELD, J., HARTL, F. U. & MOAREFI, I. (2001). Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. *Science* **291,** 1553-1557.

SONG, J., TAKEDA, M. & MORIMOTO, R. I. (2001). Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf1/ERK and cell growth. *Nature Cell Biology* **3,** 276-282.

SPRATT, D. M. (1990). The role of helminths in the biological control of mammals. *International Journal for Parasitology* **20,** 543-550.

SPRATT, D. M., BEVERIDGE, I. & WALTER, E. L. (1991). A catalogue of Australian monotremes and marsupials and their recorded helminth parasites. *Records of the South Australian Museum, Monograph Series 1,* 1-105.

SRIVASTAVA, P. K., MENORET, A., BASU, S., BINDER, R. J. & MCQUADE, K. L. (1998). Heat shock proteins come of age: primitive functions aquire new roles in an adaptive world. *Immunity* **8,** 657-665.

STANKIEWICZ, M. (1996). Observations on the biology of free-living stages of *Parastrongyloides trichosuri* [Nematoda, Rhabditoidea]. *Acta Parasitologica* **4,** 38-42.

STANKIEWICZ, M., COWAN, P. & HEATH, D. D. (1997). Endoparasitesof brushtail possums (*Trichosurus vulpecula*) from the South Island of New Zealand. *New Zealand Veterinary Journal* **45,** 257-260.

STANKIEWICZ, M., COWAN, P., JOWETT, G. H., CLARK, J. M., JOWETT, J., ROBERTS, M. G., CHARLESTON, W. A. G. & HEATH, D. D. (1997 a). Internal and external parasites of possums (*Trichosurus vulpecula*) from forest and farmland, Wanganui, New Zealand. *New Zealand Journal of Zoology*. In press.

STANKIEWICZ, M., HEATH, D. D. & COWAN, P. (1997 b). Internal parasites of possums (*Trichosurus vulpecula*) from Kawau Island, Chatham Island and Stewart Island. *New Zealand Veterinary Journal* **45,** 247-250.

STANKIEWICZ, M., COWAN, P. E. & HEATH, D. D. (1998). Endoparasites of possums from selected areas of North Island, New Zealand. *New Zealand Journal of Zoology* **25,** 91-97.

STINCHCOMB, D. T., SHAW, J. E., CARR, S. H. & HIRSH, D. (1985). Extrachromosomal DNA transformation of Caenorhabditis elegans. *Molecular and Cellular Biology* **5,** 3484-3496.

STRAHAN, R. (1983). *The Australian Museum complete book of Australian mammals*. Angus and Robertson, Sydney, Australia.

STRINGHAM, E. G., DIXON, D. K., JONES, D. & CANDIDO, E. P. (1992). Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic Caenorhabditis elegans. *Molecular Biology of the Cell* **3,** 221-233.

STRUB, A., LIM, J. H., PFANNER, N. & VOOS, W. (2000). The mitochondrial protein import motor. *Biological Chemistry* **381,** 943-949.

SU, M.-T., VENKATESH, T. V. & BODMER, R. (1998). Large- and small-scale preparation of bacteriophage lambda lysate and DNA. *Biotechniques* **25,** 44-46.

SUTO, R. & SRIVASTAVA, P. K. (1995). A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* **269,** 1585-1588.

TAKAYAMA, S., XIE, Z. & REED, J. C. (1999). An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. *Journal of Biological Chemistry* **274,** 781-786.

TANG, H. B., LIU, Y. Y., MADABUSI, L. & GILMOUR, D. S. (2000). Promoter-proximal pausing on the *hsp70* promoter in *Drosophila melanogaster* depends on the upstream regulator. *Molecular and Cellular Biology* **20,** 2569-2580.

TEMPERO, J., NEWTON-HOWES, J., RALSTON, M., SHUBBER, A., STANKIEWICZ, M. & HEATH, D. D. (1997). Life cycle of *Paraustrsrongylus trichosuri* in the brush-tailed possum. In *New Zealand Society for Parasitology Annual Meeting No 26*, Palmerston North, New Zealand.

TERBORGH, J., LOPEZ, L., NUNEZ, P., RAO, M., SHAHABUDDIN, G., ORIHUELA, G., RIVEROS, M., ASCANIO, R., ADLER, G. H., LAMBERT, T. D. & BALBAS, L. (2001). Ecological meltdown in predator-free forest fragments. *Science* **294,** 1923-1926.

THOMSON, J. & CLARK, G. (1997). Rabbit calicivirus now established in New Zealand. *Surveillance* **24,** 5-6.

THOMSON, D. M. & MEERS, J. (2001). Characterisation of an adenoirus in brushtail possums. In *Biological Management of Possums*, pp. 36-39. Ministry of Agriculture and Forestry, Wallaceville, Wellington, New Zealand.

TODRYK, S. M., MELCHER, A. A., DALGLEISH, A. G. & VILE, R. G. (2000). Heat shock proteins refine the danger theory. *Immunology* **99,** 334-337.

TORT, J., BRINDLEY, P. J., KNOX, D., WOLFE, K. H. & DALTON, J. P. (1999). Proteinases and associated genes of parasitic helminths. *Advances in Parasitology* **43,** 161-266.

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

TSUJI, N., OHTA, M. & K, F. (1997). Expression of a 70kDa heat-shock-related protein during transformation from free-living infective larvae to the parasitic stage in *Strongyloides venezuelensis*. *Parasitological Research* **83,** 99-102.

UNGEWICKELL, E., UNGEWICKELL, H. & HOLSTEIN, S. E. (1997). Functional interaction of the auxilin J domain with the nucleotide- and substrate-binding modules of Hsc70. *Journal of Biological Chemistry* **272,** 19594-19600.

VAN EDEN, W., VAN DER ZEE, R., PAUL, A. G., PRAKKEN, B. J., WENDLING, U., ANDERTON, S. M. & WAUBEN, M. H. (1998). Do heat shock proteins control the balance of T-cell regulation in inflammatory diseases? *Immunology Today* **19,** 303-7.

VAYSSIER, M., LE GUERHIER, F., FABIEN, J. F., PHILIPPE, H., VALLET, C., ORTEGA-PIERRES, G., SOULE, C., PERRET, C., LIU, M., VEGA-LOPEZ, M. & BOIREAU, P.

(1999). Cloning and analysis of a *Trichinella britovi* gene encoding a cytoplasmic heat shock protein of 72 kDa. *Parasitology* **119,** 81-93.

VELLAI, T., MOLNAR, A., LAKTOS, L., BANFALVI, A., FODOR, A. & SARINGER, G. (1999). Transgenic nematodes carrying a cloned stress gene from yeast. In *Entomopathogenic Nematodes* (ed. I. Glazer, P. Richardson, N. Boemare and F. Coudert), pp. 105-119. European Commission Publication, Luxemburg.

VIGGERS, K. L. & LINDENMAYER, D. B. (1995). The use of tiletamine and zolezepam hydrochloride for sedation or the mountain brushtail possum, *Trichosurus caninus* Ogilby (Phalangeridae: Marsupialia). *Australian Veterinary Journal* **72,** 215-216.

VIGGERS, K. L. & SPRATT, D. M. (1995). The parasites recorded from *Trichosurus* species (Marsupialia: Phalangeridae). *Wildlife Research* **24,** 311-332.

VIGGERS, K. L., LINDENMAYER, D. B., CUNNINGHAM, R. B. & DONNELLY, C. F. (1998). The effects of parasites on a wild population of the Mountain Brushtail Possum (Trichosurus caninus) in south-eastern Australia. *International Journal for Parasitology* **28,** 747-755.

VIVINUS, S., BAULANDE, S., VANZANTEN, M., CAMPBELL, F., TOPLEY, P., ELLIS, J. H., DESSEN, P. & COSTE, H. (2001). An element within the 5 ' untranslated region of human Hsp70 mRNA which acts as a general enhancer of mRNA translation. *European Journal of Biochemistry* **268,** 1908-1917.

W.H.O. (1996). The World Health Report 1996: fighting disease fostering development, pp. 43, 74. World Health Organization, Geneva.

WEDLOCK, D. N., ALDWELL, F. E. & BUDDLE, B. M. (1996). Molecular cloning and characterization of tumor necrosis factor alpha (TNF-alpha) from the Australian common brushtail possum, Trichosurus vulpecula. *Immunology and Cell Biology* **74,** 151-158.

WEDLOCK, D. N., ALDWELL, F. E. & BUDDLE, B. M. (1998). Nucleotide sequence of a marsupial interleukin-10 cDNA from the Australian brushtail possum (Trichosurus vulpecula). *DNA Sequence* **9,** 239-244.

WEDLOCK, D. N., GOH, L. P., PARLANE, N. A. & BUDDLE, B. M. (1999). Molecular cloning and physiological effects of brushtail possum interleukin-1 beta. *Veterinary Immunology and Immunopathology* **67,** 359-372.

WHEELER, G. N., HAMILTON, F. S. & HOPPLER, S. (2000). Inducible gene expression in transgenic Xenopus embryos. *Current Biology* **10,** 849-852.

WHITLOCK, H. V. (1948). Some modifications of the McMaster helminth egg-counting apparatus. *Journal of the Council for Scientific and Industrial Research* **21,** 177-180.

WILLADSEN, P., BIRD, P., COBON, G. S. & HUNGERFORD, J. (1995). Commercialisation of a recombinant vaccine against Boophilus microplus. *Parasitology* **110,** S43-50.

WILSON, M., XIN, W., HASHMI, S. & GAUGLER, R. (1999). Risk assessment and fitness of a transgenic entomopathogenic nematode. *Biological Control* **15,** 81-87.

WRIGHT, D. E. (1992). Report from the Possum and Bovine Tuberculosis Control National Science Strategy Committee. Ministry of Research Science and Technology, Wellington, New Zealand.

WRIGHT, D. E. (1993). Report from the Possum and Bovine Tuberculosis Control National Science Strategy Committee. Ministry of Research, Science and Technology, Wellington, New Zealand.

WRIGHT, D. E. (1994). Report from the Possum and Bovine Tuberculosis Control National Science Strategy Committee. Ministry of Research, Science and Technology, Wellington, New Zealand.

WU, Y., EGERTON, G., BALL, A., TANGUAY, R. M. & BIANCO, A. E. (2000). Characterization of the heat-shock protein 60 chaperonin from Onchocerca volvulus. *Molecular and Biochemical Parasitology* **107,** 155-68.

ZHENG, T., O'KEEFE, J. S., DICKIE, A., TEMPERO, J., WICKSTROM, M. & BUDDLE, B. M. (1999). Viruses for use in biocontrol of possums. In *Advances in the Biological Control of Possums*, vol. Miscellaneous Series 56 (ed. G. Sutherland), pp. 1-3. The Royal Society of New Zealand, Wallaceville, Upper Hutt, New Zealand.

ZHENG, T., DICKIE, A., LU, G. J. & BUDDLE, B. M. (2001). Marsupial herpesviruses for use in biological control of possums. In *Biological Management of Possums*, pp. 46-50. Ministry of Agriculture and Forestry, Wallaceville, Wellington New Zealand.

ZHU, X., ZHAO, X., BURKHOLDER, W. F., GRAGEROV, A., OGATA, C. M., GOTTESMAN M.E. & HENDRICKSON,. W. A.(1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272,** 1606-1614.

ZORIO, D. A., CHENG, N. N., BLUMENTHAL, T. & SPIETH, J. (1994). Operons as a common form of chromosomal organization in C. elegans. *Nature* **372,** 270-2.

ZUCCOLOTTO, P. D., HARRISON, G. A. & DEANE, E. M. (2000). Cloning of marsupial T cell receptor alpha and beta constant region cDNAs. *Immunology and Cell Biology* **78,** 103-9.