Molecular mechanisms of emerging ivermectin resistance in scabies mites from northern Australia

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Declaration

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Charles Darwin University, is the result of my own investigations, and all references to ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any other degree.

Kate E. Mounsey

14th March 2007
Abstract

Scabies has remained a worldwide problem for centuries, although its importance is frequently underestimated. It is a significant disease of children, especially in remote Aboriginal communities in northern Australia. Ivermectin has been identified as a potentially effective acaricide for mass treatment programs in scabies endemic communities, and is the treatment of choice for hyperinfested (crusted) scabies. Reports of ivermectin resistance in scabies mites raise concerns for the sustainability of such programs. It is therefore critical to define the molecular mechanisms of ivermectin resistance.

This study involved identification and characterisation of candidate genes associated with ivermectin resistance in scabies mites. Key outcomes included:

a) Identification and partial sequencing of nine ABC transporters from Sarcoptes scabiei var. hominis, five of which have been implicated in multidrug resistance in other organisms, including P-glycoprotein, previously associated with ivermectin resistance in parasitic nematodes.

b) Development of a quantitative reverse-transcriptase PCR assay to study the expression levels of candidate resistance genes in S. scabiei. Significantly, up-regulation of a delta-class glutathione-S-transferase and a multidrug resistance protein was associated with ivermectin exposure.

c) Characterisation of a novel ligand gated ion channel from S. scabiei var. hominis. The channel was shown to be modulated by pH and potentiated by ivermectin by functional expression in Xenopus laevis. Single strand conformational polymorphism analysis indicated that regions of this gene were highly polymorphic. This protein may act as the target site of ivermectin in scabies mites and therefore may be of considerable importance to the development of drug resistance.

These approaches have given us new insights into scabies mite biology and mechanisms for emerging ivermectin resistance. These may eventually assist in overcoming many of the current difficulties in monitoring treatment efficacy and allow the development of more sensitive tools for monitoring emerging resistance in the community.
Acknowledgements

Most importantly, I would like to thank my supervisors, Drs. Shelley Walton and Deborah Holt. Deb, never before have I met such a consistently optimistic and obliging person. It has been great to work with you; I hope I was not too much of a distraction! Shelley has been a fantastic mentor and role model, maintaining confidence in my abilities, even when I had lost all faith! Thanks to Dr. Ric Price and Prof. Bart Currie for their genuine interest in this project, and enthusiastic approach to research. Our collaborators at the Queensland Institute of Medical Research- A/Prof James McCarthy, Dr. Cielo Pasay, Dr. Katja Fischer and Prof. Dave Kemp have provided useful advice on many aspects of this work.

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None of this would have been possible without the continual encouragement of my friends and family. Thanks for putting up with me; I know it wasn’t always easy! Thank you Tim, for accompanying me for the better part of this journey, your support during this time in my life will always be appreciated.

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Conference presentations:


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<th>Definition</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding-cassette</td>
</tr>
<tr>
<td>ANGIS</td>
<td>Australian National Genome Information Centre</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>Ct</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DDT</td>
<td>1, 1, 1-trichloro-2, 2-bis-(p-chlorophenyl)ethane</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-amino butyric acid</td>
</tr>
<tr>
<td>GluCl</td>
<td>glutamate gated chloride channel</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-Transferase</td>
</tr>
<tr>
<td>LGIC</td>
<td>ligand gated ion channel</td>
</tr>
<tr>
<td>mdr</td>
<td>multidrug resistance gene</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance associated protein</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>SsCl</td>
<td><em>Sarcoptes scabiei</em> chloride channel</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>qRTPCR</td>
<td>quantitative reverse-transcriptase PCR</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription / transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SsCl</td>
<td><em>Sarcoptes scabiei</em> chloride channel</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
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Chapter 1

Literature Review

1.1 Introduction

Scabies is a neglected parasitic disease, and its significance is commonly underestimated. It is an infectious skin disease caused by the burrowing ectoparasitic mite *Sarcoptes scabiei*. The disease manifests as intense itching caused by allergic and inflammatory responses to the mite products. Scabies has plagued man and animals since ancient times, and despite the availability of chemotherapy, the disease remains a significant health problem, with up to 300 million people infected worldwide annually (Taplin *et al.*, 1991), although this number has been disputed (Chosidow, 2006). Relatively uncommon in developed countries, scabies remains endemic to developing regions and indigenous populations worldwide, and additionally causes problematic outbreaks in nursing homes (de Beer *et al*., 2006; Scheinfeld, 2004). Scabies is a widespread problem in many Aboriginal communities in remote northern Australia, with documented prevalence rates of up to 50% in children (Carapetis *et al*., 1997). Scabies is frequently accompanied by streptococcal pyoderma, the sequelae of which have been identified as significant causes of morbidity and premature mortality in these communities (McDonald *et al*., 2004).

1.2 History

Scabies is one of the oldest diseases known to man, and was recognised from as early as 1000BC, with references to disease symptoms in the Old Testament of Bible, and by Aristotle (Montesu and Cottoni, 1991). Until the early 17th century scabies was described as a “corruption of flesh and blood”, thought to originate from some internal illness rather than the presence of mites in the skin (Montesu and Cottoni, 1991). In 1687, Bonomo and Cestoni first described the ectoparasitic association of scabies, making it the first disease of man with a known causative agent (Lane, 1928). However, their highly significant revelation was largely ignored for nearly
200 years. In 1778, deGeer gave the first accurate description of the scabies mite, and to his credit the parasite was commonly referred to as *Sarcoptes scabiei* deGeer (Buxton, 1941). In 1868, Hebra published a well received treatise on scabies, and acceptance of the origin of this disease was finally established (Beeson, 1927).

Scabies may have been present in the natives of Papua New Guinea and New Zealand prior to European settlement (Andrews, 1976; Backhouse, 1929). In Australia however, it is probable that scabies was introduced with European settlement, since the Aboriginal population living outside these areas did not appear to be inflicted with the condition. The first reports of scabies in Aboriginal Australians are from Kittle in 1815, who noted natives suffering from the “itch” (Basedow, 1932). Scabies became increasingly prevalent during the Victorian gold rush, possibly via the migration of Chinese people in the 1850s (Lee, 1975). Between 1903 and 1927, scabies was named as one of the most common skin diseases in Australia. After this the incidence of scabies declined until World War II, when a 3-fold increase was observed (Summons, 1955). Scabies was first reported in the Northern Territory in 1942 (Kettle, 1991), likely introduced through movements of army personnel.

### 1.3 Biology of *Sarcoptes scabiei*

#### 1.3.1 Classification and determination of a single species

*Sarcoptes scabiei* belongs to phylum Arthropoda, class Acari, order Astigmata and family Sarcoptidae. The family Sarcoptidae includes *Sarcoptes scabiei*, *Notoedres cati* and *Trixacarus caviae*. The mite infests up to 40 different mammalian hosts across 17 families (Elgart, 1990). Common hosts include humans, dogs, pigs and foxes. Although *S. scabiei* mites isolated from different hosts are morphologically similar, cross infectivity studies have demonstrated they are physiologically different and largely host specific. To distinguish between mite varieties they are named according to their host species, for example, *S. scabiei* var. *hominis* (human), *canis* (dog), *suis* (pig) etc.

Traditionally it has been widely debated whether these variants represent separate species, or if one highly variable species existed. Fain (1978) undertook a study to
define the number of species and subspecies of *S. scabiei*. Variants differed in presentation of dorsal and ventro-lateral spines and in size; however there were no taxonomically significant differences between strains. He concluded that there was only one species of *S. scabiei*, but it was highly variable due to continuous interbreeding between different host-derived populations. This work was supported by later sequence analysis of ribosomal RNA, which suggested that mites from various hosts belonged to a single, albeit heterogeneous species (Zahler et al., 1999). Significantly, these studies did not include human-derived mites in their analysis, and may have been limited if based on uninformative genetic regions.

Conversely, molecular studies by Walton and colleagues (1999a) using three hyper-variable microsatellite markers demonstrated substantial genetic variation between human-derived and canine-derived *S. scabiei*, even in mites collected from the same household. Additionally, host-specific mites from geographically distinct regions were more similar to each other than to different host-derived populations in the same location. This study was later expanded to 15 microsatellite loci and two mitochondrial markers, and confirmed previous findings (Walton et al., 2004a). Limited gene flow and apparent lack of interbreeding between these populations supports designation of separate species.

1.3.2 Morphology

*S. scabiei* is a tiny mite, its ovoid body measuring 200-500 µm long and 160-420 µm wide. Adult female mites are barely visible to the naked eye but can be observed easily with microscopy. The mite is an opaque, creamy white colour with brown legs and mouthparts. The convex dorsal surface of the body is covered with numerous spines, setae and striations, and the ventral surface is flattened. The mite has no distinct head, but rather a protrusion of mouthparts beyond the anterior edge of the body known as the gnathosoma.

Adult *S. scabiei* have four pairs of legs. The first two pairs are located adjacent to the gnathosoma, and have claws or pulvilli which allow the mites to move and attach to surfaces. At the base, legs are sclerotic, exoskeletal structures called epimeres. The female mite measures 300-500 µm in length (Figure 1.1a). Legs III and IV originate on the ventral surface and end in long setae with no stalked pulvilli. The oviporus
Figure 1.1: Light microscopy images of *Sarcoptes scabiei*

a) Female *S. scabiei* var. *hominis*, dorsal view with egg (Photo: K. Mounsey)
b) Male *S. scabiei* ex wallaby, ventral view (Photo: S. Pizzutto)
consists of a transverse slit in the middle of the ventral surface. The copulatory bursa is on the dorsal side, anterior to the anus. The male mites are smaller, 200-300 µm in length (Figure 1.1b). Leg III carries a long seta, and leg IV ends in stalked pulvilli. The genital apparatus is located between the anus and fused epimeres of legs III and IV.

1.3.3 Life cycle

Historically, it has been difficult to study the passage of the mite through various life stages in detail, due to the need to treat patients and the difficulty in locating mites on the host. As a result, much of the information on the scabies life cycle has been largely anecdotal and sometimes contradictory. Much of this uncertainty was resolved by Arlian and colleagues in 1988. Using a model of New Zealand white rabbits experimentally infested with *Sarcoptes scabiei* var. *canis*, they describe egg, larval, protonymph and tritonnymph instars (Arlian and Vyszenski-Moher, 1988).

The fertilised adult female penetrates the horny layer of the skin to form a burrow. It is thought they achieve this by secreting a proteolytic saliva like substance which dissolves the host keratinocytes. This initial penetration of host skin takes less than 30 minutes (Arlian *et al.*, 1984a). There is some uncertainty as to whether the female ever leaves her burrow. Most studies suggest she does not (Burgess, 1994; Van Neste and Lachapelle, 1981), but Arlian *et al.* observed mites of all life stages leaving their burrows to wander on the surface of the skin (Arlian *et al.*, 1984a). The female begins to lay her eggs just hours after starting the burrow, and continues to lay 2-3 eggs per day for the rest of her life (around 4-6 weeks). The eggs adhere to the sides of the burrow by material secreted from ‘glue’ glands near the oviduct. It appears that very few of these eggs actually develop into adult mites (Mellanby, 1944).

The eggs hatch after about 50 hours of incubation. The larvae find their way to the skin surface to seek food and shelter in the hair follicles, where they remain for 3-4 days. They then moult into protonymphs, then tritonymphs, from which an adult male or female emerges (Arlian and Vyszenski-Moher, 1988). Because the male tritonymph is only slightly larger than the female protonymph, they were often confused and it was once thought that males only had one nymphal stage (Van Neste and Lachapelle, 1981). Following fertilisation of the female the cycle begins again.
Figure 1.2: Proposed life cycle of S. scabiei.
Development from egg to mature adult takes between 10 and 13 days (Arlian and Vyszenski-Moher, 1988).
It has been suggested that the males die following mating, but this is uncertain (Alexander, 1984).

The development from egg to adult requires 10-13 days (Figure 1.2). Other aspects of the mite life cycle, including copulation, feeding behaviour and pheremonal activity remain unclear.

1.3.4 Survival, transmission and host specificity

Mites have a thin integument and are extremely sensitive to desiccation; therefore survival off the host is highly dependant on relative humidity and temperature. In Bonomo’s letter to Redi he reports that mites can survive off the body for 2-3 days (Lane, 1928). Mites have been reported to survive up to seven days in mineral oil (Green, 1989). In general, mite survival is favoured by low temperature and high relative humidity (RH). Mellanby found that the temperature threshold for movement was 15-16°C, below which they were in a chill coma (Mellanby et al., 1942). Most rapid movement was observed at temperatures above 20°C. Arlian’s experiments found the most favourable conditions for survival were at 10°C, 97% RH. Females and nymphs survived longer than larvae and males. He also found that mites show thermotaxis, moving to higher temperatures, even if they are harmful (Arlian et al., 1984a).

The ability of mites to survive off the host has important implications for disease transmission. The role of fomites in the spread of scabies has been widely debated. Arlian found that mites held for 24-36 hours at room temperature were still capable of host penetration. All life stages penetrated the host rapidly, although developmental stages penetrated faster (Arlian et al., 1984a). However these mites were physically placed on their host by attaching mite infested skin crusts and therefore do not represent the normal mode of transmission. Nonetheless, these results advocate the potential for fomite based transmission.

The experiments of Kenneth Mellanby in the 1940s have provided fascinating insights into many aspects of the disease. Using conscientious objectors to World War II as human subjects, he studied the transmission of scabies. Experiments indicated that exchanging clothes and sleeping in beds previously occupied by infested patients failed to transmit scabies, despite intensive efforts. He found that
the disease was most commonly transmitted by skin to skin contact, and that individuals with higher mite numbers were more likely to transmit the disease (Mellanby, 1944). From this it appears that fomites are an insignificant source of transmission, except in cases of crusted scabies, where shed skin may contain enormous numbers of live mites. Transmission occurs most commonly through close personal contact with an infected person, such as embracing or sharing a bed.

Also contentious is which life stage of mite is responsible for transmission. Mellanby thought that only the newly fertilised adult female was capable of transmission and burrowing into host (Mellanby, 1944). However, Arlian showed that all life stages were capable of penetration, and that developmental stages actually penetrated faster than females (Arlian et al., 1984a). Considering that developmental stages would also highly outnumber females, it seems more feasible that they too can transmit infection.

Host specificity has been one of the most controversial issues in scabies research. Buxton notes that “biological races of *S. scabiei* which are proper to animals are not able to establish themselves on man”, and believed that they were unable to make burrows (Buxton, 1941). Studies by Walton on infected humans and dogs living in close proximity strongly support that mites are host specific (Walton et al., 1999a) (section 1.3.1). On the basis of these genetic differences, control programs in northern Australia were changed to target human scabies only. Despite this, others have observed canine mites burrowing, feeding, and laying eggs on the human host, although in self-limiting infestations (Estes et al., 1983). Previous attempts to transfer canine mites to mice, rats, guinea pigs, pigs, cattle, goats or sheep were unsuccessful; likewise human or pig mites could not be transferred to New Zealand white rabbits. Eventually, canine mites were used to successfully infest the rabbits (Arlian et al., 1984b). Notably, this represents the only animal model for scabies in the world.

From this work it has been concluded that *S. scabiei* varieties are highly host specific. Human infestations of scabies derived from other animal hosts are commonly reported; however are almost always self limiting, so it appears that mites cannot complete a life cycle away from their native host. The reasons behind host-specificity remain unclear. Arlian has found that canine mites do exhibit a degree of
host recognition behaviour, perhaps in response to temperature or odour. There may be limiting factors in the host epidermis such as specific dietary requirements (Arlian, 1989). Host immunity appears to play a role, since sensitisation to animal transmitted scabies is very different to a human infestation. (section 1.5.3).

1.4 Epidemiology of scabies

There are several reports commenting on the cyclical epidemiology of scabies, with epidemics apparently occurring every 30 years. Peaks in the incidence of scabies occurred between 1919 and 1925, 1936 and 1949, and 1964 and 1979 (Green, 1989). These peaks roughly coincided with the major wars; explaining references to scabies such as ‘camp itch’ and ‘seven year itch’ (Green, 1989). The cyclical theory is an over-simplification however, with these peaks probably more indicative of the change in social environment at the time. Furthermore, because scabies is not a reportable disease, data on prevalence is highly variable. Herd immunity has been put forward to explain the cyclical nature of scabies (Shank and Alexander, 1967); however this fails to explain its continued prevalence in developing regions.

There are many possible cofactors to scabies explored in the literature. These include seasonality, where scabies is more frequently observed in the winter months in temperate climates, and the monsoon season in the tropics (Green, 1989). This probably relates more to social factors, as these are times where people are more likely to crowd indoors. Scabies is more frequent in children, especially babies and infants (Alexander, 1984). A recent audit of two remote Aboriginal communities in northern Australia reported that 87% of children presenting with scabies have encountered the disease within the first year of life (Clucas, 2006). Scabies appears to affect both sexes equally (Green, 1989). Again, any differences associated with race and susceptibility to scabies seem to relate more to cultural and social practices rather than underlying ethnicity (Alexander, 1984). Susceptibility to scabies has been previously linked to increased frequency of human leukocyte antigen AII (Falk and Thorsby, 1981), suggesting a possible genetic factor, although this has not been associated with particular racial group.
In summary, scabies affects people of all ages, races and socioeconomic levels. It is clear that poverty and overcrowding are the two most important epidemiological cofactors. Since poor hygiene occurs concomitantly with these, it is often incorrectly labelled as a cofactor, although washing may help remove mites by physical dislodgement. The lack of influence of hygiene is demonstrated in institutions such as nursing homes, where scabies is common despite high hygiene standards (2005; Moberg et al., 1984). In remote Aboriginal communities of northern Australia overcrowding is common, with up to 30 individuals often occupying the same household (Currie et al., 1994). This is almost certainly contributing to the endemic levels of scabies, exacerbated by poor resources and inadequate medical facilities.

1.5 Clinical manifestations

1.5.1 Ordinary scabies

Often referred to as “classical” or “uncomplicated” scabies, ordinary scabies is the most prevalent form of the disease. It is caused by infestation with surprisingly few parasites, with the average number of female mites per patient less than 15, reducing with repeat infestations (Arlian, 1989; Mellanby et al., 1942). These low numbers are probably due to host immunity controlling the mite burden. Infestation commonly involves the hands, particularly the wrists and interdigital spaces (Figure 1.3a). Elbows, knees, feet and genitalia may also be affected (Chosidow, 2006).

Symptoms may vary substantially in severity, but almost always include intense pruritis, often worsening at night (Mellanby, 1977). Visible symptoms may include papular or vesicular lesions related to the site of mite burrowing, in addition to a more generalised itchy rash assumed to be part of the allergic response to the mite products (Burgess, 1994). The burrow, often regarded as the classical indicator of scabies, can be observed as a thin, greyish, line of 5-15mm (Buxton, 1941). However, burrows can be very difficult see with the unaided eye, are not always present, and are not easily located in indigenous patients (Walton et al., 2004b, personal observations). In a primary infestation of scabies, symptoms can be slow to develop, usually around 4-6 weeks. This is thought to be due to delayed immune
Figure 1.3: Ordinary scabies.
(a) Typical distribution of lesions, showing involvement of inter digital spaces.
(b) Scabies in a toddler with widespread distribution of papular lesions in soft, folded areas of skin. (Photos by B. Currie)
recognition, as sensitisation is very rapid in subsequent infestations, generally less than 48 hours (Mellanby et al., 1942). This delayed onset of symptoms contributes heavily to the spread of scabies, with people not seeking medical treatment until infestation and transmission is well established.

### 1.5.2 Scabies in children

Scabies is easily transmitted to young infants and children, probably because of increased body contact during these years. Scabies in children reflects that of adults, but has a more widespread distribution over the body, commonly involving the palms, soles, midriff, face, neck and scalp (Burgess, 1996; Orkin, 1985). This may be attributed to the mites’ predilection for soft, folded areas of skin (Gordon and Unsworth, 1945). Vesicular and papular lesions are very common (Figure 1.3b). Mellanby et al. (1942) noted a higher average number of mites in children, which probably reflects underdevelopment of the immune system.

### 1.5.3 Other forms of scabies

In addition to the manifestations described above, Orkin (1985) describes the following forms of scabies with atypical symptoms:

**Nodular scabies:** Pruritic, firm, reddish brown nodules, 5-8mm in length. These nodules typically occur in areas where skin is very thin and are more common in children. Nodules may persist for months after successful treatment. Mites are not found in nodules, making diagnosis difficult.

**Scabies in the elderly:** Inflammation of lesions may not be observed, although itching is intense. The distribution of mites may also involve the back, scalp or behind the ears. The itching is commonly misdiagnosed, incorrectly attributed to dry skin, anxiety or senility (Moberg et al., 1984). Scabies outbreaks in nursing homes are common.

**Animal transmitted scabies:** Infestation from an infected animal can be distinguished from other forms of scabies by rapid onset of sensitisation (within 48 hours) and the absence of burrows. Furthermore, areas affected reflect where direct exposure to the animal occurred. The disease is self-limiting, and removal of the animal often leads to clearing of symptoms.
Crusted scabies: An extreme form of the disease involving hyper-infestation of mites. Although it is quite uncommon, rates of crusted scabies in northern Australia are among the highest in the world (Huffam and Currie, 1998). Crusted scabies will be discussed further in section 1.6.

1.5.4 Sarcoptic mange

Scabies infestation in animals is referred to as sarcoptic mange. It affects many companion animals and livestock such as dogs, horses, pigs and camels. It has been reported in Australian populations of dingo (*Canis dingo*) (Hoyte and Mason, 1961), wild foxes (*Vulpes vulpes*) (McCarthy, 1960), wombats (*Vombatus ursinus*) (Skerrat *et al.*, 1998), and agile wallabies (*Macropus agilis*) (McLelland and Youl, 2005). Clinical manifestations may vary according to species, but generally involve raised, red papules on sparsely haired regions. As with humans, intense pruritis is experienced. If untreated, mange results in hair loss, scaling and crusting of the skin (Figure 1.4). Areas affected may include the muzzle, ears and face, legs, thighs, trunk and tail (Pence and Ueckermann, 2002). In dogs, it more commonly occurs in puppies, debilitated and older dogs, particularly when malnourished and already highly parasitized (Walton *et al.*, 2004b)(Figure 1.4a). Sarcoptic mange causes significant losses to primary industries; especially in pig herds (Davis and Moon, 1990). In southern Australia, mange in wombats is a significant cause of mortality (Martin *et al.*, 1998) (Figure 1.4b).

1.6 Crusted scabies

Crusted scabies is characterised by a proliferation of mites and formation of hyperkeratotic skin crusts (Figure 1.5). The condition was first described in 1848 by Danielson & Boeck as a variant of leprosy endemic to Norway. In 1851 Hebra correctly attributed mites to the disease, and named it “scabies norwegic boeckii” in honour of its discoverers (Alexander, 1984). This was subsequently shortened to “norwegian scabies”, a title still routinely used nowadays, despite having no inherent connection with Norway. Other proposed names have been “scabies crustosa”, “scabies keratotica” and “scabies angria” (Alexander, 1984). It 1976 it was
Figure 1.4: Severe sarcoptic mange.
(a) Extensive alopecia and skin thickening in a highly parasitized 8-10 week old puppy (Photo- K. Mounsey).
(b) Sarcoptic mange is a major cause of mortality in wombats from southern Australia (Photo- C. Willis).
deemed more fitting to describe the disease as “crusted scabies”. Crusted scabies is caused by the same mite that causes ordinary scabies, although it was once thought to be caused by a different variant, *S. scabiei* var. *crustosa* (Green, 1989). The disease was once attributed to either being derived from animals, or simply a neglected case of ordinary scabies in an insensitive patient (Buxton, 1941). However, it is now understood that progression from ordinary to crusted scabies is uncommon (Walton et al., 2004b). Moreover, many cases of ordinary scabies can be traced to an index case of crusted scabies, supporting the hypothesis that this extreme manifestation is more likely attributed to differential host immune responses.

### 1.6.1 Clinical aspects

Crusted scabies results from the host immune system being unable to control the proliferation of mites, resulting in thousands to millions of mites present on a single patient in extreme cases. As many as 6000 mites per gram of skin have been reported (Currie et al., 1995). Areas commonly affected differ to ordinary scabies and may include the soles and palms, back and buttocks (Burgess, 1996). Crusting may be widespread or localised, with severe cases affecting greater than 30% total body surface area (Royal Darwin Hospital 2006a). Buxton (1941) reports crusts being 1-2mm thick, but they can actually be much thicker, approaching 2-3cm (personal observations). Crusts contain dead skin, exudates, and mites, and their appearance varies between patients. They can be loose, soft and spongy, containing many vacant burrows, and may be easily shed. However crusts can also be extremely hard and adherent, with punch biopsies needed to reveal mites residing in the deep crusts (C. Parker, pers. comm.). In many cases pruritis may be completely absent (Alexander, 1984; Fain, 1978), but in other patients it may be extreme (personal observations).

With such extreme symptoms described, one may assume crusted scabies to be an easy diagnosis. However, the severity of symptoms may vary greatly between patients and it is often mistaken for other conditions such as psoriasis, eczema and ichthyosis (Gach and Heagerty, 2000; Gogna et al., 1985). The condition may go undiagnosed for months, especially in institutional settings (de Beer et al., 2006). Often it isn’t until a member of nursing staff develops ordinary scabies that the patient is correctly diagnosed (Moberg et al., 1984). Conversely, scabby crusts from
Figure 1.5: Manifestations of crusted scabies.
(a) Infected hyper-keratotic crusts, preceding fatal sepsis
(b) Hyperkeratosis and fissuring at joints is common.
(c) Residual depigmentation in a recurrent crusted scabies patient. (Photos- B.Currie)
infected ordinary scabies and impetigo may be mistakenly taken for crusted scabies (personal observations). Fissuring and serious secondary infections occur frequently, with five year mortality rates previously very high for crusted scabies patients (Roberts et al., 2005) (Figure 1.5a,b). Recurrent episodes of crusted scabies result in considerable skin depigmentation (Figure 1.5c), and may involve residual skin thickening, particularly on the back.

### 1.6.2 Pathogenesis

Crusted scabies usually results from underlying immunodeficiency. Predisposing conditions include substance abuse, HIV (Drabick et al., 1987), HTLV-I (Mollinson et al., 1993), systemic lupus erythematosus (SLE) (Ting and Wang, 1983), type 2 diabetes, previous leprosy and immunosupression in transplant recipients (Paterson et al., 1973). It also may be seen in patients with cognitive deficiency such as Down’s syndrome (Zakon and McQuay, 1972), or in the elderly or institutionalised who may be unable to interpret the itch (Green, 1989). Importantly, crusted scabies may also occur in persons with no known immunological deficit. A recent clinical review of 78 crusted scabies patients in northern Australia found that 42% had no known risk factor (Roberts et al., 2005). These patients appear to have a specific, as yet unknown immune deficit predisposing them to crusted scabies.

Patients generally have elevated levels of circulating antibodies, particularly IgG and IgE (Roberts et al., 2005; Walton et al., 2004b). The elevation of IgE is striking and may be over 1000 times higher than normal (Roberts et al., 2005). This dramatic, non-protective humoral response is probably due to the extreme antigenic load presented by the high mite burden. Specific antigens responsible for immune reactions include components of mite saliva and secretions, egg cases or faecal products (Arlian, 1989). Advances in scabies gene discovery (section 1.14) are helping to further elucidate scabies-specific immune responses, and specifically the differential responses of ordinary and crusted scabies patients.

Development of crusted scabies appears to involve aberrant cell mediated immunity. Histopathology studies of skin lesions showed that CD4 cells were predominant in ordinary scabies lesions, whilst infiltrates in crusted scabies were primarily CD8 (Walton, unpublished observations). However Roberts et al. (2005) found that blood
CD4 and CD8 levels and ratios are within normal limits in crusted scabies. Recent studies show elevation of the cytokine IL-4 (Walton et al., 2004b). IL-4 has been associated with preferential Th-2 type immune responses, and interestingly has been shown to stimulate keratinocyte proliferation (Yang et al., 1996). Increased IL-4 and Th-2 skewed responses have also been observed in atopic dermatitis and psoriasis (Leung, 2000; Prens et al., 1996).

1.6.3 Disease burden

Due to the incredibly high mite burden, adequate treatment of crusted scabies is challenging. Left untreated, secondary infections may lead to fatal sepsis, and prior to current treatment regimens five year mortality rates were very high (Roberts et al., 2005). Not only is this a distressing, painful and debilitating condition clinically, the psychological burden associated with crusted scabies is significant. Crusted scabies is highly contagious, and “core transmitter” patients have been recognised to contribute to burden of scabies in communities and the failure of treatment programs. Equally, because they are highly susceptible to mite infestation, crusted scabies patients are easily reinfected, and the cycle of community transmission perpetuates. For these reasons recurrent crusted scabies patients are often stigmatised, the disease commonly perceived as occurring due to neglect and poor hygiene practices.

1.7 Diagnosis of scabies

Scabies can be one of the most difficult diagnoses in dermatology. As described previously (sections 1.5, 1.6), symptoms may mimic those of other skin conditions such as eczema, psoriasis, insect bites or dermatitis. For practical purposes, diagnosis relies largely on clinical presentation and the history of the patient and their contacts. The most obvious “gold standard” for diagnosis is the identification of mites, their eggs, burrows or faeces (Burgess, 1996). Skin scrapings are performed by scraping a scalpel firmly at right angles to the skin to remove superficial layers, sometimes with the assistance of paraffin or mineral oil. Scrapings are then examined by microscopy. 10% potassium hydroxide is useful for dissolving skin and improving resolution of mites, however will also dissolve faecal pellets. This technique has very poor sensitivity due to the low numbers of mites present in ordinary scabies and the
difficulty in identifying burrows in some cases. Heukelbach & Feldmeier (2006) comment that the “sensitivity is so low that its usefulness is questionable”. Even when performed by an expert, a negative skin scraping does not exclude scabies.

Epiluminescence microscopy and videodermatoscopy have been proposed as accurate and non-invasive techniques (Argenziano et al., 1997; Lacarrubba et al., 2001), however these require specialised equipment and thus may not be suitable in a community setting. The use of a PCR-ELISA method for detecting previously undiagnosed scabies has been reported (Bezold et al., 2001), but due to the technical expertise required and low levels of S. scabiei DNA present in the skin it is not currently a viable approach.

The ideal diagnostic test for scabies would involve serological tests where the identification of mites is not required. ELISAs using whole mite extracts to detect sarcoptic mange in animal herds are commercially available. However a significant degree of variation in sensitivities between kits has been reported, and these tests are only suitable for diagnosis of infected herds, rather than individual animals (Lowenstein et al., 2004). The use of whole mite extracts may be problematic due to the heterogeneous combination of both host and parasite antigens and potential for cross reactivity (Walton and Currie, 2007). No diagnostic tests are available for human scabies, with research in this area historically impeded due to the absence of an in vitro culture system and hence limited availability of purified recombinant mite antigens. However, through the establishment of S. scabiei expressed sequence tag (EST) libraries (section 1.14), several candidate S. scabiei antigens have been reported (Harumal et al., 2003) (Mattsson et al., 2001) (Dougall et al., 2005). The ability to produce a constant supply of purified recombinant antigen, facilitating detailed in vitro studies, suggests a highly specific diagnostic test for scabies may be a real possibility in the near future.

1.8 Scabies in northern Australia

Although Australia is one of the most developed countries in the world, conditions in remote Aboriginal communities often more closely resemble those of a “third-world” country. Many diseases long eradicated from urbanised areas remain highly
CHAPTER 1

problematic to indigenous populations. Diseases disproportionately higher in Aboriginal children include skin infections, upper respiratory tract infectious, intestinal nematodes, urinary tract infections, diarrhoeal disease and trachoma (Currie, 2005). Factors contributing to this increased burden of disease are numerous, and their interactions complex, including inadequate housing infrastructure (Bailie and Runcie, 2001), overcrowding, poor sanitation (Gracey et al., 1997), and limited access to medical resources.

1.8.1 Prevalence

Rates of scabies and skin infections in Aboriginal communities are extremely high, their occurrence second only to respiratory infections (Clucas, 2006). Scabies is a relatively recent disease of Aboriginal Australians, believed to be introduced through white colonisation (section 1.2). Conversely other endemic skin conditions such as tinea corporis are thought to have been introduced much earlier from South East Asia via Macassan trading (Green and Kaminski, 1977). The prevalence rates of scabies reported in northern Australian Aboriginal communities are up to 50% in children and 25% in adults (Carapetis et al., 1997; Fraser, 1994), with major increases observed in the 1990’s (Currie et al., 1994). In a recent clinical audit undertaken in two communities, 73% of children presenting to clinics had been infected with scabies at least once (Clucas, 2006). Findings also revealed that the greatest burden of scabies was in the very young, with 63% of these children presenting in their first year, and a median presentation age of 4.2 months (Clucas, 2006). Similar observations have been reported in other scabies endemic regions such as the Solomon Islands (Lawrence et al., 2005). Children are in close contact with many carers, and thus are good indicators of the burden of scabies in a given community.

1.8.2 Health impact

Skin infections (pyoderma) often occur concomitantly with scabies (Figure 1.6). Scabies lesions (often exacerbated by excoriation) serve as an entry point for pathogenic bacteria, primarily Group A Streptococcus (GAS; Streptococcus pyogenes) with secondary colonisation by Staphylococcus aureus (Currie and Carapetis, 2000). In northern Australian communities scabies is reported to underlie 50-70% of streptococcal pyoderma in children (Carapetis et al., 1997). The sequelae
Figure 1.6: Infected scabies lesions.
Scabies lesions provide an entry point for pathogenic microorganisms such as Group A *Streptococcus*. (Photos- K. Mounsey, B. Currie).
from GAS pyoderma in these communities is significant, including acute post-
streptococcal glomerulonephritis, and acute rheumatic fever (ARF). Rheumatic heart
disease (RHD) in particular is a significant cause of premature mortality (Carapetis et al., 1999). Rates of RHD in Aboriginal populations in north Australia are four-fold
higher than in other developing countries, and the death rate is 30.2 per 100,000, in
stark contrast to 1.1 per 100,000 in non-Aboriginal Australians (McDonald et al., 2004).

ARF is traditionally only thought to be associated with streptococcal pharyngitis,
however most of the epidemiological data supporting this is derived from more
temperate regions where GAS skin infection is not common (McDonald et al., 2004).
Significantly, symptomatic pharyngitis is seldom reported, and GAS throat carriage
is low in northern Australia (Carapetis et al., 1997; Carapetis et al., 1999; McDonald et al., 2004). The apparent link between GAS skin infection and RHD has lead to
cooncerted efforts to reduce the rates of scabies and subsequent skin infections in
Aboriginal communities.

1.8.3 Community control programs

Control programs in northern Australia are currently based on mass treatment with
the topical acaricide 5% permethrin (section 1.9). These initiatives are based on the
highly successful programs implemented in Panama, where scabies is also endemic
(Taplin et al., 1991). The Panama model involved the supervised treatment of
everyone in the community regardless of infestation. Rates of scabies decreased from
33% to 1.5%, and this reduction was sustained with continued surveillance over
many years (Taplin et al., 1991). A modified form of this intervention program was
initially trialled in two northern Australian communities, with prevalence of scabies
in adults decreasing from 25% to 6% in one community, and sustained up to two
years later (Carapetis et al., 1997). In the second study, prevalence rates decreased
from 35% to less than 5%, before slowly increasing again twelve months post-
intervention (Wong et al., 2002).

Treating single communities in isolation is unlikely to lead to sustainable decreases
due to the highly transient nature of people between communities. To successfully
control scabies in northern Australia, initiatives are required on a regional scale.
Such a program was introduced in 2004, with the launch of the East Arnhem Healthy Skin program—a collaborative effort including the Menzies School of Health Research, Cooperative Research Centre for Aboriginal Health and Australasian College of Dermatologists. It involves approximately six communities in the north east Arnhem region (Figure 1.7). The program has a multidisciplinary approach, involving regular screening for scabies, tinea and skin infections; annual mass treatment for scabies; education and environmental measures. There is a strong focus on community ownership and education, with the eventual objective for self-sustaining programs to be implemented within the community.

Figure 1.7: The east Arnhem region of the Northern Territory. The East Arnhem Healthy Skin program aims to reduce the burden of scabies and skin sores in six Aboriginal communities in this region.
Current reports indicate these mass treatment programs have successfully reduced prevalence of scabies and skin sores, however rates quickly return to baseline levels in the months following treatment (Clucas, 2006). Possible explanations for this current lack of sustainability may include waning levels of community enthusiasm, difficulties in follow-up and early detection of scabies. A major difference between the north Australian and Panama models is that treatments are not supervised in the former. A recent community survey indicated that many people, even community health workers, were unaware of correct application method for topical permethrin (O'Connor, 2006). Inadequate treatment of core-transmitter crusted scabies may also be contributing to the limited success of mass treatment in some areas (section 1.6.3).

1.9 Treatment for scabies

Although scabies is one of the oldest known diseases to man, there are surprisingly few effective treatments available today. Treatment for scabies involves the application of topical acaricides, although oral ivermectin is becoming increasingly popular (section 1.10). Regardless of the acaricide used, there are three important principles governing scabies therapy:-

1) Treatment of the patient- it is critical that the topical acaricide be applied to the entire body, including under the nails, and that it is left for the recommended time. The majority of treatment failures are attributed to incorrect application. Additionally, because most acaricides are not ovicidal, re-treatment may be necessary in some cases.

2) Treatment of all potential contacts- a frequent cause of recurrent scabies is reinfection from untreated contacts. Diagnosis of infection and therefore treatment is complicated by the delayed onset of symptoms, therefore it is essential that all contacts are treated regardless of symptoms.

3) Treatment of surroundings- although *S. scabiei* can only live off the host for a limited period of time (section 1.3.4), it is still advised to treat surroundings, particularly in more severe cases of scabies. Although acaricidal sprays can be employed, general cleaning and laundering of items at high temperatures (60°C) is usually adequate (NT CDC, 2003b).
When selecting the most appropriate treatment for scabies, there are several factors to consider. In developing regions, cost and availability of the acaricide are important. Ideally, the drug should be easy to apply, minimally absorbed by the skin, non-toxic, effective against both mites and eggs, and effective as a single dose regimen. From the most widely used treatments described below, we see that no one drug currently fulfils all these criteria.

1.9.1 Sulphur

Sulphur compounds have been used as acaricides for centuries, and are still a relevant option in certain cases today. In northern Australia, sulphur was still used on babies until quite recently (Connors, 1994). It is generally used as a 2-10% precipitate in a petrolatum base. Usually 6% ointment is preferred (Karthikeyan, 2005). It is considered safe for pregnant and lactating women, and for infants younger than two months (Roos et al., 2001). Interestingly, Mellanby (1942) found that sulphur itself is not toxic to mites. Rather, the ointment reacts with the skin to form toxic by-products which are acaricidal. This is an important consideration if including sulphur ointment for in vitro studies. Although effective and inexpensive, sulphur compounds are messy to use, smelly and sometimes irritating (particularly in tropical climates) (Rees, 1985). Furthermore, multiple applications are often required for successful treatment. Therefore, sulphur has largely been abandoned for more ‘user friendly’ alternatives.

1.9.2 Crotamiton

10% crotamiton ointment has been used as an acaricide since 1946 (cited in Roos et al., 2001). It has antibacterial, antiparasitic and antipruritic activity, which coupled with its low-toxicity, makes it a popular option for children. However, it is probably the least reliable acaricide, with Taplin et al. (1991) reporting a cure rate of only 60%. Resistance to crotamiton has also been reported (Roth, 1991). It is the currently recommended treatment in northern Australia for babies less than two months of age (CARPA, 2003a). For successful treatment, multiple applications are required, with best results obtained when applied twice daily for five consecutive days (Karthikeyan, 2005; Roos et al., 2001). These factors suggest that crotamiton may not be ideal for controlling infant scabies in the community setting, especially considering the high burden of scabies in this group.
1.9.3 Benzyl Benzoate

Benzyl benzoate has been employed for its acaricidal properties since 1900 (Mellanby et al., 1942), and remains widely prescribed today. It is an ester of benzoic acid and benzyl alcohol, obtained from balsam of Peru (Karthikeyan, 2005). At a concentration of 25% it is highly efficacious, both in vivo and in vitro (Walton et al., 2000). Unfortunately this concentration can cause severe skin irritation, particularly in children. Consequently it often needs to be diluted, reducing its efficacy and possibly creating potential for resistance. In France, 10% benzyl benzoate combined with 2% sulphur is the most widely prescribed topical acaricide (Buffet and Dupin, 2003). It is generally not recommended for infants and pregnant women due to its allergenic potential. Despite this, it was used as a first line treatment in Aboriginal communities prior to the widespread introduction of 5% permethrin (Connors, 1994). Buffet and Dupin (2003) claim benzyl benzoate is effective against ova, although there is a lack of data to support this. Treatment guidelines for this drug also vary, with some recommending three applications within 24 hours (Roos et al., 2001). Given its extreme potency in vitro, this seems excessive. Some authors suggest this treatment has fallen into disrepute (Karthikeyan, 2005; Roos et al., 2001), however since this is one of the few acaricides where resistance is not a concern to date, its use today is still very much relevant.

1.9.4 Lindane

Until recently, lindane (gamma benzene hexachloride) was one of the most commonly used medications for scabies worldwide. It is a potent lipophilic insecticide first used for scabies in 1948 (Wooldridge, 1948). In vitro sensitivities show a similar efficacy to benzyl benzoate (Walton et al., 2000), although in vivo lindane appears to be slightly less effective. Potential neurotoxicity associated with lindane use has been a lingering concern, leading to its withdrawal from the market in Australia and many European countries (Chosidow, 2006). Adverse effects reported include numbness, cramps, dizziness, seizures and even death (Roos et al., 2001). The neurotoxic effects of lindane poisonings resemble that of related insecticides such as DDT (Davies et al., 1983). Toxicity is believed to occur through increased subcutaneous absorption, with infants and the elderly at particular risk (US FDA, 2003c). To minimise absorption, it is advised that lindane be applied to cool,
dry skin, and not immediately after taking a bath. It is important to note that most side effects have been attributed to inappropriate application (Purvis and Tyring, 1991). Despite these issues, lindane remains as a first or second line treatment choice in many countries. With concern growing however, more widespread restrictions on its usage are likely in the near future (Wooltorton, 2003).

1.9.5 Permethrin

Permethrin is a synthetic pyrethroid first marketed in 1977 (Meinking, 1996). Originally used in an agricultural setting, it has been available for scabies for about 20 years, over which time its use has steadily increased in popularity. For scabies, permethrin is applied topically at a concentration 5%. Permethrin has potent insecticidal activity, but low toxicity and is very well tolerated by most. Unlike lindane, permethrin is rapidly metabolised in the skin by esterases, and less than 1% is absorbed (Karthikeyan, 2005). When applied correctly cure rates of over 90% for ordinary scabies have been observed, reportedly more efficacious than lindane or crotamiton (Meinking, 1996; Purvis and Tyring, 1991; Taplin et al., 1990).

Permethrin has now replaced lindane as the first line treatment for scabies in Australia, the United Kingdom and the United States (Buffet and Dupin, 2003). It has also been successfully implemented for community treatment of scabies, but concerns have been raised regarding the emergence of drug resistance as a result of such treatment protocols (section 1.11.2). One of the few caveats of permethrin is that it is the most expensive topical acaricide, often restricting its use in the developing regions that need it the most (Karthikeyan, 2005).

1.9.6 Novel therapeutics

From the above it can be seen that there are few acaricides available today that are safe, simple and effective. Furthermore, with emerging drug resistance a very real consideration, development of novel acaricides would undoubtedly be of benefit. Several natural agents with acaricidal properties have been described, including lippia oil (Lippia multiflora) (Oladimeji et al., 2000), camphor oil (Eucalyptus globulus) (Morsy et al., 2003), and pastes of tumeric (Circuma longa) and neem (Azadirachta indica) (Charles and Charles, 1992). Although high cure rates (97%) were obtained with the latter, neem was found to have little acaricidal properties in vitro (Walton et al., 2000).
One promising new treatment is tea tree oil. Derived from *Melaleuca alternifolia*, tea tree oil is a traditional Aboriginal medicine commonly used for skin infections and insect bites. This essential oil has demonstrated antimicrobial activity (Carson and Riley, 1995), however its potential as an antiparasitic had not been explored until recently. In vitro studies found that at a concentration of 5%, tea tree oil had excellent acaricidal properties. Terpinen-4-ol was identified as its most potent active ingredient, with this component alone killing 85% of mites within the first hour of exposure— much more rapidly than permethrin and ivermectin (Walton *et al.*, 2004c). In current treatment protocols for crusted scabies at Royal Darwin Hospital, benzyl benzoate ointment is supplemented with 5% tea tree oil (Royal Darwin Hospital, 2006a). Not only is this a potent combination in vitro, but the soothing properties of tea tree oil reportedly help reduce the extreme irritation experienced with benzyl benzoate (B. Currie, unpublished observations). However, more data regarding the safety and in vivo efficacy of tea tree oil via clinical trials are required before its widespread promotion as a novel therapeutic agent for scabies can occur.

**1.10 Ivermectin**

**1.10.1 Pharmacokinetics & safety**

Ivermectin (22, 23 di-hydro avermectin) was first identified in the mid-1970s during the screening of Japanese soil samples by the Merck Corporation in collaboration with Kisato Institute (Richard-Lenoble *et al.*, 2003). This discovery heralded an unprecedented new era in parasite control for both veterinary and human medicine.

Ivermectin is a semi-synthetic, chemically modified avermectin, derived from the fermentation products of the actinomycete *Streptomyces avermitilis* (Burkhart, 2000). It is a member of the macrocyclic lactone group, related to macrolide antibiotics, but with antibiotic properties itself. Other related, commonly used avermectins include abamectin, emamectin benzoate, doramectin and selamectin. These all have the same basic structure (Figure 1.8) but vary in pharmacokinetic properties.

Extensive studies have been performed on the pharmacokinetics of ivermectin in many species. Ivermectin is highly lipophilic and rapidly absorbed. In humans, it
reaches peak plasma levels within five hours of ingestion. In humans, the ivermectin half-life in plasma has been measured to be between 12-28 hours and the drug is excreted almost entirely through faeces (Burkhart, 2000; Ottesen and Campbell, 1994; Roos et al., 2001). The half-life in humans is shorter than most other animals (Cerkvenik-Flajs and Grabnar, 2002). The bioavailability and half-life are influenced by several factors, including formulation, administration and stomach contents. In animals the pharmacokinetics also vary substantially according to species, sex, age and physiological status (reviewed in Cerkvenik-Flajs and Grabnar, 2002). Absorption of ivermectin is reportedly improved on an empty stomach (Roos et al., 2001), however recent investigations demonstrate increased plasma concentration when the drug is administered with food (Guzzo et al., 2002) (Currie, unpublished) (Figure 1.9).

The correlation between ivermectin serum levels and efficacy is not straightforward. For example, although the serum half-life of ivermectin is less than one day in some animals, it may remain in tissue stores for prolonged periods of time. Ivermectin is presumed to be delivered to the scabies mite via ingestion of intraepidermal fluids (Burkhart, 1999). Therefore, the bioavailability of the drug in the skin is an important consideration. To be effective against scabies in a single dose, the drug must be retained in tissue for a sufficient period for the mites to emerge from the eggs (about 2 days, see section 1.3.3). A number of studies have demonstrated that ivermectin does indeed reach the skin at therapeutic concentrations (Baraka et al., 1996) (Scott and McKellar, 1992). In humans, peak levels in the skin were achieved within eight hours, and declined markedly after 24 hours. Concentrations were higher in oilier areas of the skin such as the forehead, in concurrence with its lipophilicity and retention in fatty tissue (Haas et al., 2002).

There appears little information regarding the distribution of ivermectin in hyperkeratotic skin crusts and dermis, although this is of considerable importance to treatment efficacy for crusted scabies. The relatively the short half-life of ivermectin in humans suggests that at least two doses may be required for treatment of scabies, particularly if many eggs are present. Other avermectins used for sarcoptic mange, such as doramectin, have a substantially longer half-life, and therefore may be more efficacious than ivermectin in a single dose regimen for animals (Voyvoda et al., 2005).
Ivermectin has a very wide margin of safety in most mammals, although there are major differences in sensitivity between species. Primates for example are far less sensitive than rodents. Ivermectin can be toxic to certain breeds of dogs from the collie lineage (Paul et al., 1987). This has been linked to specific mutations in the P-glycoprotein encoding \textit{mdr1} gene, enabling ivermectin to cross the blood-brain barrier and enter the central nervous system (Neff et al., 2004; Roulet et al., 2003) (section 1.13.1.1). In over twenty years of use in filariasis control, severe adverse effects reported for ivermectin are minimal. Common side effects include gastrointestinal disturbances, headache, fever, dizziness and pruritis (delGiudice et al., 2003).

Figure 1.8: Chemical structure of ivermectin.
Ivermectin is a semi-synthetic avermectin derivative, belonging to the macrocyclic lactone group.
Figure 1.9: Time-course of circulating ivermectin concentration in the plasma of a crusted scabies patient.
Note the dramatic increase following dose 2, administered immediately following a fatty meal, compared with dose 1, given on an empty stomach (Currie 2004, pers. comm).

Adverse effects to higher (800 µg/kg) doses of ivermectin were recently reported for onchocerciasis patients in Cameroon (Kamgno et al., 2004). Treatment of loiasis with ivermectin may be associated with severe adverse reactions in patients with high microfilaremia, especially when coinfected with *Onchocerca volvulus* (Gardon et al., 1997). Many of these adverse effects in filariasis have been associated with an inflammatory type ‘Mazzotti’ reaction caused by the death of microfilaria, rather than from the drug itself. This is evidenced by the reduction in symptom severity with decreased microfilaria load (Kamgno et al., 2004). It has been postulated that the bacterial endosymbiont *Wolbachia* contributes to this post treatment inflammatory reaction (Hise et al., 2004), although not all filarial nematodes are infected (McGarry et al., 2003). Recent studies indicate that *S. scabiei* in northern Australia are not infected with *Wolbachia* (Mounsey et al., 2005). It is important to mention that there are also a small number of unexplained severe adverse effects involving CNS impairment, which are not consistent with an acute immunological type reaction. It has been suggested that P-glycoprotein deficiencies at the blood-
brain barrier may be involved, although this has not yet been investigated (R Prichard, pers comm.)

There have been very few side effects reported for ivermectin therapy of scabies. Barkwell et al. (1997) reported an association of ivermectin with deaths in the elderly, but this was refuted by several groups at the time (Coyne and Addiss, 1997; Diazgranados and Costa, 1997; Reintjes and Hoek, 1997), and a true correlation seems unsubstantiated. Coyne et al. (1997) makes the interesting point that these patients were treated with other acaricides, including lindane, prior to ivermectin therapy, so it is difficult to separate the effects of these drugs, especially when the risks of using lindane on the elderly are established (section 1.9.4).

Due to the possibility of blood-brain barrier underdevelopment causing ivermectin toxicity, the use of ivermectin is currently contraindicated in children under 15kg and in pregnant and lactating women (Burkhart, 1999). Despite these cautions, ivermectin has been used in these groups with no adverse effects (Gyapong et al., 2003). Studies on the excretion of ivermectin in breast milk are limited, but appeared to differ significantly between lactating animals (Cerkvenik-Flajs and Grabnar, 2002). In humans, the few studies done indicate the concentration of ivermectin in breast milk is minimal. Ogbuokiri et al. (1993) comment that the potential dose received by the infant is so small that exclusion of breast-feeding mothers from mass ivermectin treatment may be unnecessary. No studies report a negative effect of ivermectin during pregnancy (Chippaux et al., 1993; Gyapong et al., 2003; Pacque et al., 1990) but due to a lack of data, this group remains excluded in ivermectin treatment guidelines.

There have been several randomised controlled trials on the use of ivermectin in children, but due to inconsistencies between methodologies conclusions are difficult to make. Addis et al. (1997) reported increased fever, headache, myalgias and cough in children receiving ivermectin with or without albendazole, compared to the placebo or albendazole alone groups, but adverse effects were mild and well tolerated. Brooks & Grace (2002) compared ivermectin and benzyl benzoate in the treatment of scabies in children. They found no serious adverse effects with either treatment, although benzyl benzoate caused more local skin irritation as would be expected. Marti et al. (1996) reported more abdominal and chest pain with
ivermectin than with albendazole, but these effects were short lived. It is important to note that most of these studies were not considering scabies specifically, so adverse effects in these children may be related to nematode infestation. More data on the safety of ivermectin in children under 15kg needs to be collected.

1.10.2 Medical applications

Ivermectin has been used widely in veterinary and agricultural settings for nearly three decades. It was first introduced to veterinary practice in 1981 (Geary, 2005), quickly becoming the agent of choice due to its broad spectrum, high potency, and persistence. Its availability in a variety of formulations meant associated labour costs were minimal.

The first applications of ivermectin in human medicine came in the late 1980s, for treatment of filarial diseases. For many years the use of ivermectin was limited to mass treatment campaigns for control of onchocerciasis, a significant public health problem in west Africa (Richard-Lenoble et al., 2003). Since 1988, over 100 million doses of ivermectin have been distributed under a free donation program scheme initiated by Merck, and continuing today under the African Program for Onchocerciasis Control (APOC) (www.apoc.bf). Ivermectin also has potent microfilaricidal activity against other filarial nematodes such as Brugia malayi, Wuchereria bancrofti and Loa loa. It is also effective against strongyloides, cutaneous larva migrans and many intestinal nematodes.

Although used for the treatment of sarcoptic mange in animals for many years (Dourmishev et al., 2005), ivermectin is a relatively new treatment for human scabies. After seeing reduced ectoparasite burdens in those treated for filariasis (delGiudice and Marty, 1999), interest grew regarding its potential as a new therapeutic agent for scabies.

Ivermectin is the only oral acaricide, which has obvious advantages with ease of application simplifying treatment supervision and increasing patient compliance. It was initially envisaged that single dose ivermectin would replace topical creams entirely, greatly simplifying treatment of this difficult disease (Burkhart et al., 1997; Lawrence et al., 1994). However this has not yet occurred, and more than ten years later topical therapy remains the mainstay of scabies treatment. The drug has been
approved in France, the Netherlands and Mexico for the treatment of ordinary scabies (excluding pregnant and lactating women and children <15kg), but remains unlicensed in most other countries, including Australia (except for crusted scabies) (Chosidow, 2006; delGiudice et al., 2003). There are still many questions regarding ivermectin therapy for scabies, including the optimal number of doses, interval between doses and drug concentration.

Several studies report the efficacy of ivermectin as a single dose for ordinary scabies. Meinking et al. (1995) documented a 100% cure rate one month after single dose ivermectin, although at two weeks after treatment this was only 45%. A single 150 µg/kg dose was used for controlling a scabies outbreak in an African prison, with 95% cure rate after two months (Leppard and Naburi, 2000). In a study in Tahiti, Glaziou et al. (1993) found ivermectin to be equally effective as benzyl benzoate, with a 70% cure rate after one month. However, both drugs were administered in suboptimal doses (100 µg/kg ivermectin, 10% benzyl benzoate). Chouela et al. (1999) compared lindane to ivermectin, with one month cure rates comparable (95% vs 96%). Patients with more severe cases of scabies in both groups needed a second treatment. In a comparison between 5% permethrin and ivermectin, Usha et al. (2000) found permethrin to be more efficacious, with 97.8% cured compared to only 70% with ivermectin, although after a second dose of ivermectin the two were comparable.

Only one study failed to see any response to ivermectin treatment, although numbers were very small, and the authors acknowledged this may be due to reinfestation prior to follow-up (Dunne et al., 1991). 200 µg/kg appears to be the most widely used concentration, as levels below this may result in a reduction in efficacy. Higher doses (400 µg/kg) have also been used (Bockarie et al., 2000), but this still did not prevent reinfestation.

Several of the above studies indicate that a single dose of ivermectin may be inadequate for many cases of scabies. Given its relatively low residual activity, a second treatment after seven days seems practical to kill the newly hatched mites (Burkhart, 1999; Meinking et al., 1995). However, for practical purposes, a single dose ivermectin regime is seen as important for successful community implementation. Recently, Lawrence et al. (2005) reported the success of ivermectin
mass treatment of scabies in the Solomon Islands. All residents received a single 160-250 µg/kg dose, with children under 15kg treated with permethrin. Scabies prevalence rates dropped from 25% to less than 1%, and remained low for many months after the intervention. These results suggest that ivermectin may hold promise as a new tool in the mass treatment of scabies, although its higher cost when compared to topical therapies may limit its use in some areas.

Ivermectin is a popular choice for institutional settings and for crusted scabies, where topical application is difficult and may not adequately penetrate the thick crusts (Huffam and Currie, 1998). Although not formally licenced for scabies treatment in Australia, it has been approved for compassionate use in crusted scabies. Most crusted scabies patients require multiple doses of ivermectin, although single doses have been used successfully in children with crusted scabies recalcitrant to topical therapy (Patel et al., 1999). Crusted scabies is extremely difficult to treat, with no general consensus on optimal treatment strategies and very few comparative studies published. Alberici et al. (2000) compared ivermectin and benzyl benzoate in HIV associated crusted scabies. They found neither drug to be effective when used in isolation, and that combination therapy was the best option. Similarly, the history of ivermectin treatment for crusted scabies patients in northern Australia strongly advocates combination treatment and multiple doses of ivermectin (section 1.11.3). At Royal Darwin Hospital, treatment for severe crusted scabies has increased to up to seven 200 µg/kg doses of ivermectin over four weeks, combined with keratolytic and topical therapy (usually benzyl benzoate supplemented with 5% tea tree oil) (2006a). Milder cases involve three doses of ivermectin. Despite these comprehensive measures, treatment failures have been reported (section 1.11.3).

1.10.3 Mode of action

Ivermectin causes paralysis in parasites by binding to ligand gated chloride channels, causing an influx of negatively charged chloride ions, resulting in hyperpolarisation of synapses and paralysis (Roos et al., 2001). Early investigations suggested that γ-aminobutyric acid (GABA) gated chloride channels were the primary target of ivermectin. Avermectins were shown to affect inhibitory neuromuscular transmission and interneurons in Ascaris. This effect was partially reversed by picrotoxin (Kass et al., 1980). Since GABA is the primary neurotransmitter in invertebrate somatic
musculature, and picrotoxin is a known GABA-channel blocker, this was a reasonable assumption. In mammals, ivermectin acts as an agonist of GABA receptors in the central nervous system (Nobmann et al., 2001). Studies by Holden-Dye et al. (1990) also supported the interaction of ivermectin and GABA-gated chloride channels on the paralysis of *Ascaris*. A recent report by Feng et al. (2002) provided functional evidence that ivermectin potentiated a GABA-A receptor in *Haemonchus contortus* (section 1.13.2.2).

In arthropods, ivermectin causes ataxia and paralysis. This reaction is quite distinct to that of other insecticides. Injection of avermectin into cockroaches blocked skeletal muscle contraction and nerve cord activity. Interestingly, avermectin suppresses the action of lindane, suggesting that both drugs interact with GABA-gated chloride channels, but at distinct sites and with markedly different effects (Wafford et al., 1989). GABA is an inhibitory neurotransmitter, so blocking the channel by lindane results in hyper-excitability, and potentiation by avermectin causes increased inhibition, and therefore paralysis.

It became apparent that the GABA-gated chloride channels may not be the sole target of ivermectin toxicity in invertebrates. The first evidence of this was by Duce & Scott (1985), who showed that ivermectin increased chloride conductance in locust muscle bundles insensitive to GABA, suggesting that another type of chloride channel may also be involved. This study also showed ivermectin blocked muscle responses to ibotenic acid, which is known to activate glutamate-gated chloride channels. An important study by Geary et al. (1993) found that minute amounts of ivermectin (0.1nM) paralysed pharyngeal pumping in the nematode *H. contortus*, whereas the somatic musculature was far less sensitive, with greater than 10nM ivermectin required to reduce motility. In nematodes, pharyngeal musculature are sites of primarily glutamergic neurotransmission, whereas somatic musculature is associated with GABA-ergic transmission (Holden-Dye and Walker, 1990). In 1994, a glutamate-gated chloride channel (GluCl) was cloned for the first time from *Caenorhabditis elegans* (Cully et al., 1994). Ivermectin activated these channels strongly and irreversibly at the low concentrations expected to produce paralysis. Cloning of a homologous gene from *Drosophila* confirmed channel sensitivity to ivermectin (Cully et al., 1996). Many other studies have demonstrated high affinity ivermectin binding to native and recombinant glutamate gated chloride channels.
from nematodes and arthropods (Cheeseman et al., 2001; Dent et al., 1997; Forrester et al., 2003; Kane et al., 2000).

Despite these momentous research efforts, there are still many ambiguities concerning ivermectin activity in nematodes. For instance, the relative importance of the pharyngeal versus somatic sites of ivermectin activity in vivo is not fully resolved (reviewed in Sangster et al., 2005). Although pharyngeal muscle is far more sensitive to ivermectin (Geary et al., 1993), other studies on H. contortus found that ivermectin did not significantly inhibit feeding in vivo (Sheriff et al., 2005). It is possible that if paralysis of movement occurs more rapidly than pharyngeal paralysis, albeit at higher concentrations, than this may be sufficient to allow digestive clearance and removal of the worm. (Sheriff et al., 2005).

Although the significant role of GluCls on ivermectin toxicity in nematodes is well established, far less is understood regarding ivermectin activity in arthropods. It is now becoming increasingly apparent that ivermectin can interact with a broad range of chloride channels. Interestingly, a native Drosophila ivermectin receptor was recently proposed to contain both GABA and GluCl subunits co-expressed (Ludmerer et al., 2002). Ivermectin sensitive histamine gated chloride channels have been discovered (Gisselmann et al., 2002), along with several new ligand gated ion channel subunit clades in Drosophila which are not represented in nematodes (Dent, 2006). Finally, Schnizler et al. (2005) has identified a novel chloride channel from Drosophila which is pH sensitive and also activated by ivermectin. These recent findings highlight the substantial diversity and complexity in the arthropod ligand gated ion channel family, and show that much work is required before we can begin to understand the interaction of ivermectin with target sites in arthropods.

1.11 Acaricide resistance in scabies: clinical and in vitro observations

Most treatment failures for scabies can be attributed to incorrect application of the acaricide, or failure to treat all contacts leading to reinfestation. However, there are now increasing reports of treatment failures linked with drug resistance. Of particular concern is the potential emergence of resistance to the two acaricides widely used in
northern Australia—permethrin and ivermectin. Thus questions are being raised regarding the future efficacy of these agents in this scabies endemic region. In addition to this, resistance to other acaricides such as lindane and crotamiton have also been reported worldwide (Roth, 1991).

1.11.1 Lindane resistance

Treatment failures with lindane were reported as early as 1983 (Hernandez-Perez, 1983). Several cases of resistance to 1% lindane have been reported in the United States and central America. In many of these cases, treatment was supervised and thus treatment failure could not be attributed to incorrect application of the acaricide (Hernandez-Perez, 1983; Meinking, 1996; Purvis and Tyring, 1991; Roth, 1991; Taplin, 1983). In northern Australia mites obtained from a crusted scabies patient were still alive after six hours in vitro exposure to lindane (Woltman, 1994), (Fraser, 1994). However a later in vitro study by Walton et al. (2000) found all mites to be dead within three hours of lindane exposure, suggesting that resistance may be limited to isolated cases. Molecular mechanisms for lindane resistance in scabies have not been investigated, although since lindane acts on GABA-gated chloride channels (section 1.10.3), target site mutations may be involved, similar to the Rdl type mechanism observed in other insects (section 1.13.2.2). Despite these concerns regarding resistance, lindane has been used successfully in millions of cases, and was still a reliable first-line treatment until its recent withdrawal in many countries, including Australia due to neurotoxicity concerns (section 1.9.4).

1.11.2 Permethrin resistance

Permethrin resistance in other ectoparasites such as head lice is widespread, with clinical failures to 1% permethrin now reported in Australia, Israel, England, France, and the Czech Republic (Witkowski and Parish, 2002). This suggests that emerging permethrin resistance in scabies is a real possibility. Clinical resistance of scabies mites to permethrin is yet to be documented, although anecdotal reports of failure in remote communities receiving mass treatment are increasing (B Currie, pers. comm.). Longitudinal studies conducted in northern Australia region confirm increasing in vitro tolerance. In 1994, before widespread permethrin was introduced, all mites were killed within 30 minutes of in vitro exposure in permethrin (Fraser, 1994; Woltman, 1994). By the year 2000 however, 35% of mites were alive after
three hours of exposure, and a significant proportion remained alive overnight (Walton et al., 2000). Interestingly, a population of *S. scabiei* var. *canis* maintained on rabbits now appear to have developed resistance after many years of permethrin exposure (Arlian, unpublished).

Mechanisms for pyrethroid resistance in arthropods are well established, and include:
1) Mutations to the voltage sensitive sodium channel, commonly known as *kdr* (knock-down resistance); 2) Increased enzymatic degradation by esterases (e.g. carboxylesterase B1); and 3) Enzymatic degradation by other detoxification enzymes such as the cytochrome P450 and Glutathione S-Transferases (David et al., 2005; Guerrero et al., 2002; He et al., 1999b; Lee et al., 2000). Recently a genotyping strategy was developed to survey for sodium channel mutations in *Sarcoptes scabiei* (Pasay et al., 2006). Studies on the permethrin tolerant *S. scabiei* var. *canis* have identified a *kdr* type mutation not present in permethrin naïve mites, but this has not been identified in any var. *hominis* populations to date (Pasay, unpublished).

1.11.3 Ivermectin resistance

Ivermectin was first used in northern Australia in April 1992. Early observations were not striking, with two doses of ivermectin having a negligible effect on overall mite burdens in severe crusted scabies (Currie et al., 1994). In another report, a single 240 µg/kg dose of ivermectin, daily keratolytic therapy and multiple permethrin doses failed to resolve infestation, with multiple live mites observed after two weeks (Currie et al., 1995). Considering that these early failures may have been due to inadequate penetration of ivermectin into skin crusts, a three dose regimen was introduced in 1996 with greater success. Unfortunately relapses to this were reported soon after, and in one case live mites were observed 19 days after commencement of therapy (Huffam and Currie, 1998). A second report confirmed that three doses were inadequate for severe crusted scabies, with live mites observed some 48 days after the first dose, and 19 days after the third dose of ivermectin. Microsatellite genotyping studies undertaken indicated this treatment failure was not due to external re-infection but was more likely caused by recrudescence (Walton et al., 1999b). Importantly however, sensitivity assays performed during this time suggested mites were still ivermectin sensitive in vitro (Walton et al., 2000). In light of these clinical failures, treatment guidelines were amended in 1998 to a five dose
ivermectin regimen. This still proved to be clinically inadequate, with more relapses reported, and monthly prophylaxis unsuccessful at preventing reinfestation (B. Currie, pers. comm).

In 2000, the clinical failure of ivermectin was reported in two recurrent crusted scabies patients who had received multiple doses of ivermectin over a five year period (Currie et al., 2004). The first case occurred in January 2000 in a 36 year old female who had received 17 doses of ivermectin in the twelve months preceding, and 30 doses since 1995. Live mites were observed after a month of multiple doses of ivermectin, and in vitro testing showed mites with significantly increased tolerance to ivermectin (Figure 1.10). The second case occurred in August 2000, in a 48 year old male who had received 58 doses of ivermectin since 1996. Despite receiving seven 270 µg/kg doses of ivermectin, numerous live mites were observed 26 days after commencement of therapy. Furthermore, upon presentation three weeks after the seventh dose, the condition had worsened substantially. Again, these clinical observations were supported by in vitro assays showing apparent ivermectin resistance, with some mites surviving overnight ivermectin exposure (Figure 1.10). Eventually both cases were cleared by increasing topical therapy. To our knowledge, these are the first reports of ivermectin resistance in human disease, and thus represent cases of international significance.

Although to date these cases appear to be isolated events, this emergence of resistance and the requirement for increasing ivermectin doses certainly raises concerns regarding the usefulness of ivermectin for the treatment of scabies especially in severe and recurrent cases.
Figure 1.10: In vitro resistance of *S. scabiei* to ivermectin.
Kaplan-Meier survival curve showing mites obtained from two crusted scabies patients with clinical ivermectin failure in 2000, compared to ivermectin sensitive mites collected in prior years. P=<0.0001 (Currie et al., 2004).

### 1.12 Ivermectin resistance in other organisms

Ivermectin resistance first appeared in *H. contortus* from sheep and horses, only 33 months after its introduction (Shoop, 1993). These early cases of resistance originated in South Africa in the mid to late-1980s (Carmichael et al., 1987), with further reports emerging from Brazil (Echevarria and Trindade, 1989) and New Zealand (Watson and Hosking, 1990). The first documentation of ivermectin resistance in Australia came from a Western Australian property in populations of *Ostertagia circumcincta* (Shoop, 1993). After nearly thirty years of intensive use, ivermectin resistance is now widespread in veterinary practice (Wolstenholme et al., 2004). Anthelmintic resistance in livestock has serious economic implications with the threat to the sheep industry in Australia a pertinent example (Besier and Love, 2003).

Of note, there have not been any reports of ivermectin resistance in heartworm control (Geary, 2005), with selection for resistance in *Dirofilaria immitis* apparently low (Prichard, 2005). Although there have been no definitive cases of ivermectin resistance in human filariasis, recent reports of suboptimal responses are concerning (Awadzi et al., 2004; Osei-Atweneboana et al., 2005). An examination of these
persistent microfilaridermias suggests that adult female worms may be becoming resistant to ivermectin (Awadzi et al., 2004).

Avermectins have been used extensively since 1990 for the control of arthropod pests in agricultural settings. Resistance to abamectin in the two-spotted spider mite, *Tetranychus urticae* has been reported in several locations in the United States (Beers et al., 1998; Campos et al., 1995), the Canary Islands, and Holland (Campos et al., 1996). Emerging field resistance to abamectin has also been reported in other species, such as the tomato leaf miner (*Tuta absoluta*) (Siqueira et al., 2001), and persea mites (*Oligonychus perseae*) (Humeres and Morse, 2005). Moderate levels of abamectin resistance have been reported in the colorado potato beetle (*Leptinotarsa decemlineata*) (Argentine and Clark, 1990). Laboratory selection studies generated extremely high levels of abamectin resistance in house flies (*Musca domestica*) (Scott et al., 1991), suggesting that if avermectins were introduced for domestic pest control, resistance could occur rapidly (Clark et al., 1994).

### 1.13 Ivermectin resistance mechanisms

There are several factors determining the emergence of ivermectin resistance. (Wolstenholme et al., 2004). Selection pressure has a major impact on the rate and distribution of resistance development. This may be influenced by the pharmacokinetic properties of the drug, the concentrations used, the number of treatments, and the employment of alternative therapeutic drug classes. Coles et al. (2005) recently demonstrated that when high ivermectin selection pressure is applied to *H. contortus*, resistance occurs rapidly. Similar ease and rapidity of selection has been reported for some arthropods (Argentine and Clark, 1990; Konno and Scott, 1991).

The biology of the parasite in question is another obvious determinant. Development of resistance may be influenced by life cycles, as parasites with complex or indirect lifecycles seem to be slower to develop resistance (Sangster and Gill, 1999). The genetic diversity of the parasite prior to drug selection may also be involved, as an increased diversity also increases the likelihood of resistance alleles existing in a population. Genetic factors, such as the number of genes responsible for resistance,
and whether alleles are dominant or recessive, also contribute (reviewed in Wolstenholme et al., 2004). Genetic studies on ivermectin resistance in nematodes such as *H. contortus* suggest that inheritance of resistance is dominant (Le Jambre et al., 2000), however most reports in arthropods concur that resistance is autosomal, incompletely recessive, and polygenic (Argentine et al., 1992; Konno and Scott, 1991; Liang et al., 2003; Siqueira et al., 2001).

What does this mean for drug resistance in scabies? Scabies mites have a short, direct life cycle which may favor resistance development. In ordinary scabies, reproductive success and resulting mite populations are relatively low due to host immunity, but the opposite is true for crusted scabies. Furthermore, microsatellite studies indicate substantial genetic heterogeneity, with up to 46 alleles at a particular locus reported in a single population of mites (Walton et al., 1999b). Ivermectin has a relatively short half-life in humans, thus mites would not be exposed to sub-therapeutic drug concentrations for prolonged periods. Again, the exception of this may be in crusted scabies, where drug concentrations in hyperkeratotic skin crusts are likely to be sub-therapeutic, favoring selection for resistant mites, particularly under a multiple dose regimen. Overall, this suggests that selection for resistance in crusted scabies could occur rapidly, as demonstrated by previous reports (section 1.11.3), however the judicious use of treatments may help to circumvent this.

Despite many years of intensive research in nematodes, the molecular mechanisms of macrocyclic lactone resistance are yet to be clearly defined. It now increasingly apparent that the genetic basis of ivermectin resistance in nematodes and arthropods is complex, multifactorial and appears to vary even between closely related species (Clark et al., 1994; Wolstenholme et al., 2004). Despite this complexity, several candidate mechanisms are well-established. These include: 1) Increased drug efflux, mediated by transporter proteins such as P-glycoprotein; and 2) Target alteration mediated by changes to glutamate or GABA gated chloride channels. Selection at a β-tubulin gene suggesting alteration to neuronal amphids has also received recent attention (Eng et al., 2006). Additionally, arthropod studies advocate a role for metabolic mechanisms, that either inactivate or eliminate the drug. The following section will briefly attempt to review what is known about avermectin resistance in nematodes and arthropods.
1.13.1 ABC Transporter mediated efflux

ATP-binding cassette (ABC) transporters are plasma membrane proteins capable of transporting a large range of substrates from cells. ABC transporters represent the largest family of transport proteins, and make up about 5% of the *Escherichia coli* genome (Sheps *et al*., 2004). They perform a variety of transport functions, but are most well known for their roles in drug resistance. Although there are variations, ABC transporters generally possess a similar functional structure consisting of two highly conserved cytoplasmic ATP-binding domains, and two transmembrane domains, each containing six transmembrane segments. These two domains, which are homologous, but not identical, are joined by a cytoplasmic linker, often referred to as the “P-loop” (Figure 1.11). The ATP-binding domains contain consensus sequences common to all ABC transporters, known as the Walker A & B motifs, which are separated by about 100 amino acids, plus an additional ABC signature motif (Dean *et al*., 2001).

ABC transporters are grouped into subfamilies according to amino acid sequence similarity and domain organisation. To date eight different ABC transporter subfamilies have been described in the literature, and these are well conserved across species (Table 1.1). The number of genes present in any given subfamily may be stable in some cases. Subfamilies E & F for example encode highly conserved housekeeping proteins that contain the signature ATP-binding regions, but lack transmembrane domains and thus do not function as transporters (Dean *et al*., 2001). Other families have undergone extensive expansion and contraction. The P-glycoprotein family (ABC-B) in *C. elegans* for example has undergone massive duplication with respect to the human and *Drosophila* genomes; and the ABC-H family appears to have been lost in higher eukaryotes (Table 1.1) (Sheps *et al*., 2004).

ABC transporters are excellent candidates for drug resistance, due to their conservation across all orders, and association with multidrug drug resistance in most of these. At least three subfamilies of ABC transporters have been implicated in mammalian drug resistance (Dean *et al*., 2001). In relation to ivermectin resistance, P-glycoproteins (subfamily B) and multidrug resistance proteins (subfamily C) are of particular interest.
Figure 1.11: Structural organisation of a “typical” ABC transporter.
Two transmembrane domains (1-6, 7-12), and two ATP-binding domains (NBFs), separated by a cytoplasmic linker region (figure originally adapted from www.med.rug.nl/mdl).

Table 1.1: ATP-binding cassette subfamilies in human, Drosophila and C. elegans genomes

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Common names</th>
<th>Number of genes</th>
<th>Human</th>
<th>Drosophila</th>
<th>C. elegans</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ABC1</td>
<td>12</td>
<td>19</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>MDR/Pgp</td>
<td>11</td>
<td>10</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>MRP/CFTR</td>
<td>12</td>
<td>12</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>ALD</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
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<tr>
<td>F</td>
<td>GCN20</td>
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<td>3</td>
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<td>White</td>
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<td>H</td>
<td>0</td>
<td>3</td>
<td>2</td>
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</tr>
</tbody>
</table>

1 (Dean et al., 2001)
2 (Roth et al., 2003)
3 (Sheps et al., 2004)
1.13.1.1 P-glycoprotein

P-glycoprotein is a 150-170kD protein, discovered by Juliano & Ling (1976) as an overexpressed protein in multidrug resistant cell lines from Chinese hamster ovary. In 1986 the 4kb gene encoding P-glycoprotein was cloned and referred to as the multidrug resistance gene, or mdr1 (Ueda et al., 1987). Most research on P-glycoproteins has focused on their ability to confer chemotherapeutic resistance in cancer cell lines. P-glycoproteins have a wide substrate specificity, including antimicrobials, anticancer agents, steroid hormones, HIV-protease inhibitors, and immunosuppressants (reviewed in Wang et al., 2003). Further investigations found P-glycoprotein to be normally expressed in mammalian excretory or barrier tissues, such as the blood-brain barrier (Cordon-Cardo et al., 1989), intestinal epithelia (Li et al., 1999), placenta (Lankas et al., 1998), testis (Melaine et al., 2002) and renal tubules (Hori et al., 1993). From this it has been proposed that P-glycoproteins normal physiological function involves the absorption, distribution and excretion of xenobiotics, thus protecting vital organs from “toxic insult” (Wang et al., 2003).

P-glycoproteins have been implicated in drug resistance in several parasitic protozoa. In malaria parasites, mutations in the P-glycoprotein homologue pfmdr1 were originally implicated in chloroquine resistance (Foote et al., 1990), however other studies suggested the effect of pfmdr1 alterations on chloroquine resistance are minor. For example, overexpression of the pfmdr1 homologue, pgh1, was not required for chloroquine resistance (Cowman et al., 1991). Where mefloquine resistance is concerned, evidence supporting pfmdr1 involvement is stronger, with increased copy number associated with mefloquine, and possibly with artesunate resistance (Price et al., 1999; Price et al., 2004; Reed et al., 2000). P-glycoproteins have also been associated with drug resistance in Leishmania (Jones and George, 2005).

Ivermectin is known to be an excellent substrate for P-glycoprotein transporters. The first evidence for this came in what the authors describe as a “serendipitous discovery” (Schinkel et al., 1994). A group of laboratory mice were inadvertently killed when treated with ivermectin for a mite infestation. Closer analysis revealed that only experimental mice carrying a deletion in the mdr1a gene were affected. Toxicity resulted from P-glycoprotein deficiency, thus permitting ivermectin to cross
the blood brain barrier and enter the central nervous system. Similarly, ivermectin hypersensitivity is known to occur in dogs of the collie lineage naturally carrying a 4bp mdr1 mutation that introduces a premature stop codon in the P-glycoprotein gene (Neff et al., 2004; Roulet et al., 2003).

The association of P-glycoprotein and ivermectin resistance has been most intensively studied in the nematode H. contortus. Early studies were very promising, showing increased mRNA and altered restriction patterns in P-glycoprotein of ivermectin selected strains. Furthermore, application of the P-glycoprotein inhibitor verapamil increased ivermectin efficacy (Xu et al., 1998). In contrast to this, Smith & Prichard (2002) found no evidence of P-glycoprotein up regulation in H. contortus. Several studies have since reported allelic polymorphism indicative of ivermectin selection in H. contortus (Blackhall et al., 1998a; Le Jambre et al., 1999; Sangster et al., 1999), and more recently in Onchocerca volvulus (Ardelli et al., 2005a; Eng and Prichard, 2005). Despite this genetic evidence of P-glycoprotein involvement in ivermectin resistance, there is still lack of functional studies that substantiate these findings.

Support for a possible role of P-glycoproteins in ivermectin resistance in arthropods comes from the effect of verapamil on the mosquito Culex pipiens, where the addition of the drug resulted in a reduction of the LD50 of ivermectin by 50% (Buss et al., 2002), reflecting results obtained for Chironomus larvae (Podsiadlowski et al., 1998). This suggests that if selected for, P-glycoprotein could possibly confer a degree of resistance to multiple classes of insecticides. To date there has been no functional evidence supporting efflux mediated drug resistance in arthropods, however molecular studies on P-glycoprotein in arthropods are virtually non-existent.

1.13.1.2 Multidrug resistance proteins

Closely related to P-glycoproteins are the ABC-C group of transporters; commonly known as multidrug resistance proteins (MRPs). MRPs were first discovered in 1992, in multidrug resistant lung cancer tumour cells not over-expressing P-glycoprotein (Cole et al., 1992). In humans, there are six members of the MRP family, with MRP1 & 2 the best characterised (Borst et al., 1999). Structurally, MRPs are similar to P-glycoproteins, but with several distinguishing characteristics. Many MRP members
are larger (190kDa), and possess a third N-terminal transmembrane domain. Alignment of the N-terminal ATP binding domain shows that MRPs lack 13 amino acids between the Walker A and B domains. Additionally, unlike P-glycoproteins, the linker region between ATP-binding domains is poorly conserved in MRPs (Borst et al., 1999).

Like their P-glycoprotein counterparts, MRPs have a broad, sometimes overlapping substrate specificity. They can extrude both lipophillic uncharged molecules and water soluble anionic compounds (Lespine et al., 2005). Importantly, they often transport substances conjugated with glutathione. This strong affinity for glutathiolated substrates suggests that MRP function may be closely associated with glutathione transferases (Roth et al., 2003). In humans, MRP genes are associated with multidrug resistance to anti-cancer drugs such as doxorubicin, daunorubicin, vincristine, and colchicines (Dean et al., 2001).

The association of MRPs with drug resistance has been investigated in several parasitic species. In C. elegans, mrp1 knockouts are hypersensitive to toxic pigments and heavy metals (Broeks et al., 1996). LtPgpA, an MRP type transporter from Leishmania, has been associated with resistance to antimonials and arsenic (Legare et al., 2001). Similarly, overexpression of an MRP in Trypanosoma brucei is correlated with high levels of arsenical resistance (Shahi et al., 2002). Members of the MRP family have recently been identified in arthropods. In Anopheles gambiae, MRP-type transporters constitute the largest subfamily of ABC transporters (Roth et al., 2003). Interestingly, the human MRP1 homologue in D. melanogaster and A. gambiae is alternatively spliced to at least 12 isoforms (Graillies et al., 2003; Roth et al., 2003), a phenomenon not previously seen in other MRPs. The implications of this are not yet known, but since exon variation occurs in regions apparently involved in substrate recognition, this protein may broadly impact multidrug resistance in arthropods.

Although most reports to date focus solely on P-glycoprotein mediated ivermectin efflux, more recent evidence suggests a role for MRPs also. It was once proposed that P-glycoprotein was the only pump for ivermectin (Gottesman et al., 1996). At the human blood brain barrier, application of leukotrine C4 (an MRP substrate / inhibitor) did not influence ivermectin transport, suggesting that MRP was not...
actively transporting ivermectin at this site (Miller et al., 2000; Nobmann et al., 2001). However, it has been recently demonstrated that MRPs also interact with ivermectin. In cell-lines over-expressing MRP1, ivermectin inhibited the transport of MRP substrates, showing that ivermectin is both a substrate for, and inhibitor of MRPs, although at a lower affinity than P-glycoproteins (Lespine et al., 2005). In further support for a role of MRPs in ivermectin resistance, several non P-glycoprotein transporters from *O. volvulus* were recently found to show selection and a reduction in genetic variability after treatment with ivermectin (Ardelli et al., 2006; Ardelli and Prichard, 2004).

### 1.13.2 Ligand gated chloride channels

Members of the ligand gated ion channel (LGIC) family mediate neurotransmission in muscles and neurons. Subunit proteins of this family have a consistent topology of an extracellular ligand binding domain, and four transmembrane domains. These assemble to form a heteropentameric structure (Figure 1.12), although little is known about the native assembly of subunits. It is probable that native subunit composition varies between different species, perhaps even different tissues, which makes elucidating their contributions to resistance a complex undertaking (Wolstenholme and Rogers, 2005).

![Figure 1.12: Basic structural organisation of a ligand gated chloride channel.](image)

Each subunit comprises four membrane spanning segments and an N-terminal extracellular domain forming the ion selective pore (Bloomquist, 2003).
Ivermectin is known to interact with multiple members of the ligand gated chloride channel family (section 1.10.3). It is therefore highly likely that alterations to these channels that change ivermectin binding affinity may be associated with development of resistance. To date most attention has centred around the glutamate gated chloride channels, although there has recently been a renewed interest in the GABA channels.

1.13.2.1 Glutamate gated chloride channels

Simultaneous mutation of three *C. elegans* GluCl α-subunits, (avr-14, avr-15, and glc-1) confers extremely high level resistance to ivermectin (approx 4000-fold). Mutation of only one of these genes however conferred either no or very modest sensitivity, suggesting that multiple genes may independently contribute to ivermectin sensitivity and resistance (Dent *et al.*, 2000). In *D. melanogaster*, a proline to serine substitution C-terminal to the M2 region (P299S) in the glc1 gene conferred 3-fold resistance to ivermectin in native preparations. When this mutation was expressed in *Xenopus laevis* oocytes, the effect was more dramatic, with a 14-fold reduction in ivermectin sensitivity (Kane *et al.*, 2000). However, caution should be taken when interpreting such results, as lab induced mutations may not accurately reflect the emergence of ivermectin resistance in the field.

By performing single strand conformational analysis using a GluClα fragment from *H. contortus*, Blackhall *et al.* (1998b) found significant changes in allele frequencies in three independently selected strains. One allele in particular increased in frequency, suggesting that ivermectin was exerting selection pressure on the subunit. However, when additional *H. contortus* subunits were investigated, no amino acid changes were identified in resistant strains (Hejmadi *et al.*, 2000). Despite this, further evidence of GluCl involvement comes from the nematode *Cooperia oncophora*. Ivermectin resistant field isolates contain several mutations in the GluClα extra-cellular domain, with expression in *Xenopus* oocytes confirming that one of these mutations (L256F) resulted in a modest, but significant (2.5-fold) decrease in both glutamate and ivermectin sensitivity (Njue *et al.*, 2004).

Another possibility is that resistance may be mediated by a change in the number of binding sites rather than a direct genetic mutation. Hejmadi *et al.* (2000) and Paiement *et al.* (1999) describe an increase in glutamate binding in resistant strains,
CHAPTER 1

suggesting that an up regulation of GluCl may lead to a decrease in ivermectin toxicity. Phenotypically this may be substantiated, with resistant *H. contortus* apparently less sensitive to ivermectin toxicity on pharyngeal pumping (Kotze, 1998). Similarly, radioligand binding studies showed a decrease in abamectin binding in highly resistant house flies (Konno and Scott, 1991).

### 1.13.2.2 GABA gated & other novel chloride channels

Other members of the ligand gated chloride channel family also interact with ivermectin, but their contribution to resistance remains unclear. Ivermectin resistant *H. contortus* shows allelic selection at a GABA-gated chloride channel gene, with the resistance associated allele differing by 4bp (Blackhall *et al.*, 2003). When expressed in *Xenopus*, this allele confers altered ivermectin binding, with increased sensitivity to GABA and attenuation of GABA responses associated with resistance, and potentiation of GABA in the susceptible allele (Feng *et al.*, 2002).

In arthropods GABA channel mutations are well known to confer resistance to cyclodiene insecticides such as dieldrin (ffrench-Constant *et al.*, 1993). Interestingly, Kane *et al.* (2000) found that *Rdl* flies highly resistant to dieldrin were also 3.3-fold cross resistant to ivermectin. Other studies do not support the occurrence of abamectin cross resistance in arthropods (Argentine and Clark, 1990). The possibility of ivermectin receptors in *Drosophila* containing co-expressed *Rdl* and GluCl subunits (Ludmerer *et al.*, 2002) does however suggest a possible association of GABA-gated chloride channel genes in ivermectin resistance in arthropods.

The identification of additional classes of ligand gated chloride channels that also interact with ivermectin adds a new degree of complexity to the situation (section 1.10.3) (Dent, 2006; Iovchev *et al.*, 2002; Schnizler *et al.*, 2005). Although no association with ivermectin resistance involving these subunits has been demonstrated to date, available data on their better known LGIC counterparts make them worthy of further exploration.

### 1.13.3 Metabolic detoxification

A common mechanism for insecticide resistance is increased metabolic degradation mediated via detoxifying enzymes such as cytochrome P450s, carboxylesterases, and glutathione S-transferases. Although these mechanisms have not been explored with
ivermectin specifically, several studies concerning metabolic resistance to the related insecticide abamectin exist. An excellent review of this early research is provided by Clark et al. (1994). These studies have focused on three main arthropods: the colarado potato beetle (*Leptinotarsa decemlineata*); the house fly (*Musca domestica*); and the two-spotted spider mite (*Tetranychus urticae*). Most research has concentrated on the effect of metabolic synergists on abamectin toxicity in laboratory selected resistant strains, and also biochemical assays with metabolic substrates to determine which pathways are involved. Support for the involvement of all three major resistance associated metabolic pathways exists to varying degrees.

Results with the synergist piperonyl butoxide, and increased cytochrome P450 levels implicate monoxygenase-mediated abamectin detoxification in the colarado potato beetle (Argentine et al., 1992) and the diamondback moth (*Plutella xylostella*) (Liang et al., 2003). A role has also been suggested in two spotted spider mites (Campos et al., 1996; Stumph and Nauen, 2002). Conversely, this mechanism does not appear to be of significance to abamectin resistant house flies (Argentine et al., 1992). Carboxylesterases also appear to play a role in abamectin resistance in some arthropods. In the colarado potato beetle, the esterase synergist DEF resulted in moderate reduction of resistance, and esterase levels were significantly elevated (Argentine et al., 1992). Elevated carboxylesterases were also observed in the tomato leaf miner (*Tuta absoluta*) (Siqueira et al., 2001). Studies on two spotted spider mites suggest only a minor role (Campos et al., 1996; Stumph and Nauen, 2002).

Glutathione S-transferase (GST) mediated detoxification has been deemed to play only a minor role in abamectin resistance in most arthropods (Clark et al., 1994). The major exception to this are two-spotted spider mites, which are of considerable interest due to their similarity to scabies mites. Early studies showed that application of diethyl maleate, a GST inhibitor, significantly increased abamectin toxicity in resistant mites (Campos et al., 1996). Stumph and Nauen (2002) confirm this, with pretreatment of resistant strains with diethyl maleate reducing resistance by nearly 10-fold. Additionally, a 6-11 fold increase in GST activity in resistant strains was recorded in a fluorometric assay using monochlorobimane. Interestingly, when selection pressure was removed and abamectin resistance lost, GST levels returned to “normal”. Other studies looking at GST activity in ivermectin resistance are less convincing. No interaction was observed between ivermectin and recombinant GST
from the tick *Boophilus microplus* (da Silva Vaz *et al.*, 2004). However, it is not known which class of GST was examined, as not all classes are implicated in insecticide resistance.

Despite these suggestions of metabolic involvement in arthropods, similar studies on nematodes are limited. In one study, Kotze (1998) tested the effect of metabolic pathway inhibitors on *H. contortus* ivermectin sensitivity, with no significant result. However no effect was observed with P-glycoprotein inhibitors either, even though P-glycoproteins have been extensively linked to ivermectin resistance in *H. contortus*. Subsequently, it is difficult to draw conclusions from such studies. In contrast, there is a decided lack of information on molecular mechanisms of abamectin resistance in arthropods. Therefore, when considering candidate mechanisms in the scabies mite, it is important that all “camps” are visited.

### 1.14 Scabies gene discovery

Until recent years, there had been little progress in understanding the molecular biology of *S. scabiei*, primarily because of limitations in obtaining sufficient genetic material. By utilizing the shed, mite-infested skin of crusted scabies patients, Walton and colleagues were able to overcome this problem and initiated the first ever DNA-based studies on scabies in the mid 1990’s (Walton, 1999). This led to important discoveries regarding population genetics and host specificity of scabies, and influenced control programs (section 1.3.4).

This work was rapidly expanded to involve the construction of highly informative *S. scabiei* var. *hominis* cDNA libraries (Harumal *et al.*, 2003). A large scale project to sequence 50,000 cDNA clones was initiated in 2001, as a collaboration between the Menzies School of Health Research, the Queensland Institute of Medical Research and the Australian Genome Research Facility (Fischer *et al.*, 2003a; Fischer *et al.*, 2003b). In addition to this, a *S. scabiei* var. *vulpes* cDNA library has been constructed from fox mites (Ljunggren *et al.*, 2003). In both libraries, a high number of ESTs (47.5%) showed no significant homology to other sequences in GenBank and thus appear to represent novel sequences. From these libraries, a number of biologically relevant molecules have been identified, including genetic markers, candidate immunodiagnostic molecules, vaccine candidates and potential allergens.
(reviewed in Walton et al., 2004b). Notably, several ESTs potentially involved in drug resistance were also discovered, facilitating the work presented in this thesis.

1.15 Consequences of acaricide resistance

The emergence of acaricide resistance is a serious threat to the control of scabies. There are very few effective drugs currently available for scabies, and the development of new drugs is unlikely in the near future. Scabies is a ‘neglected’ disease, with most afflicted from poor, developing regions, far removed from the lucrative drug research and development market. Although there may be potential for immunological control, any vaccine or other immunotherapy would be years, perhaps decades away. Therefore, it is essential to prolong the life of the limited available drugs if we are to achieve sustainable control of this disease.

Once resistance is established in populations, management is difficult. If we rely on clinical evidence of resistance, it will be too late to control it, as resistance alleles will already be established in the population. Assessment of drug efficacy in scabies is currently based on clinical reports and / or in vitro drug sensitivity studies, which can be costly, labour intensive and time consuming. Furthermore, because most scabies patients have fewer than 10 mites, in vitro studies are only possible for cases of crusted scabies and therefore their application is limited. The development of molecular based methods would enable much greater sensitivity, as genetic changes associated with resistance could be detected before they are established. Molecular tools have been developed and applied to monitor for parasitic drug resistance in many medical, veterinary, and agricultural settings (for examples see Guerrero et al., 2001; Sangster et al., 2002), however little work has been undertaken in human scabies.

1.16 Objectives of this project

In light of emerging ivermectin resistance in human crusted scabies, and the probability of ivermectin being employed for more widespread community control programs, there is an urgent need to identify mechanisms of ivermectin resistance in
the scabies mite. The genetic basis for ivermectin resistance must first be defined to facilitate the design of molecular approaches to enable more effective monitoring and control of the spread of resistance in scabies mites endemic to northern Australian Aboriginal communities. This project proposed to undertake a focused study to identify the targets and mechanisms of ivermectin resistance in *S. scabiei* var. *hominis*.

Where arthropods are concerned, and especially in arachnids, there is very little existing molecular information regarding putative ivermectin resistance genes. Based on candidate mechanisms from other organisms, we developed the following hypotheses to explore potential ivermectin resistance mechanisms in *S. scabiei*:

a) Ivermectin resistance is due to alterations to members of the ligand gated chloride channel family (e.g. Glutamate and/or GABA gated chloride channels);

b) Ivermectin resistance is a result of ABC transporter mediated efflux pumps (e.g. P-glycoproteins and/or multidrug resistance proteins), and

c) Ivermectin resistance occurs through increased metabolic degradation mediated by detoxification enzymes (e.g. Glutathione S-transferases).

### 1.17 Contributions to this thesis

The work presented in this thesis was conducted by myself, with the following main exceptions. In vitro sensitivities were performed by various members of the scabies and skin pathogen research laboratory at the Menzies School of Health Research, over the ten year period investigated. The *S. scabiei* var. *hominis* genomic DNA library was constructed by Dr. Deborah Holt at the Menzies School of Health Research, and Dr. Holt also provided assistance with screening for the genomic ligand gated chloride channel (Chapter 5). The functional characterization of the *Sarcoptes scabiei* chloride channel in *Xenopus* oocytes (Chapter 5) was performed in collaboration with Associate Professor Joseph Dent (McGill University, Montreal, Canada)
Chapter 2

General Methods

2.1 Ethical Approval

Ethical approval for this study was obtained from the Charles Darwin University Human Research Ethics Committee (reference H03061); and the Human Research Ethics Committee of the Northern Territory Department of Health and Community Services and Menzies School of Health Research (project number 01/15). Written informed consent was obtained from patients before any samples were collected. This process involved the use of plain language statements and scabies information flipcharts to educate patients about the study prior to signing of the consent form. All work involving genetic manipulation was approved by the Darwin Regional Institutional Biosafety Committee and the Office of the Gene Technology Regulator.

2.2 Mite collection

Skin scrapings were obtained from patients admitted to the Royal Darwin Hospital diagnosed with crusted scabies. Skin crusts were collected from either the bedding of the infected patient or by gently scraping skin crusts from the body. Skin scrapings were transported to the laboratory and placed in glass petri dishes on a heating block at 28°C. This encouraged *Sarcoptes scabiei* var. *hominis* to move down towards the heat source. The dish was inverted, removing excess skin but leaving mites attached to the plate via their ambulacral pulvilli. Samples were inspected for mites using a dissecting microscope (40x magnification) and mites separated from skin using a probe and forceps if necessary. If numerous live mites were found in the sample, in vitro drug sensitivity assays were conducted (section 2.3). For long-term storage, mites were collected in eppendorf tubes and stored at -80°C. Additional mites were collected from crusted scabies patients in remote communities as part of the East Arnhem Healthy Skin Project. Scrapings and mite collection were performed as
described above, except a heating block was not used, and samples were refrigerated and transported to the laboratory within 48 hours of collection.

2.3 In vitro drug sensitivity assays

Mites were tested within three hours of collection from the patient wherever possible as per methods described in (in Walton et al., 2000) Acaricides tested represented the most commonly used treatments in northern Australia (Table 2.1) and were used within expiry dates. In general, 10 single mites per treatment were tested. The emulsifying ointment BP88 which has no active acaricidal components was used as a negative control and to dilute acaricides.

Using a cotton swab, a 30mm petri dish was lightly coated with approximately 0.1g of the product to be tested. Care was taken to ensure even coverage and that sides and lid of the dish were also coated. A single mite was carefully placed onto the centre of the dish, with the viability of the mite checked immediately after transfer. Dishes were incubated on a heat block at approximately 28°C. If assays were continued for longer than 3 hours or overnight a damp cloth was placed over the dishes to maintain relative humidity. Mites were generally assessed at 15, 30 and 60 minutes, and at hourly intervals thereafter (Table 2.2). Mite status was recorded as walking (active movement), slow (movement in one spot), paralysed (movement of legs or pharynx only when touched with probe), or dead. Time of death was recorded and mites stored in individual tubes. All sensitivity results were recorded on the laboratory database. Survival data was analysed and Kaplan-Meier survival curves produced using Prism V3.0.2 (GraphPad Software Inc). Statistical comparison of survival curves were made using the log-rank test. An assay was considered valid if survival curves obtained with experimental acaricides were significantly different to those obtained with the BP88 negative control.
### Table 2.1: Acaricides used in in vitro sensitivity testing

<table>
<thead>
<tr>
<th>Acaricide</th>
<th>Retail name, components and working concentration</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP88 (negative control)</td>
<td>Emulsifying ointment B.P (50% soft white paraffin, 30% emulsifying wax, 20% liquid paraffin)</td>
<td>Sigma (Castle Hill, NSW)</td>
</tr>
<tr>
<td>Benzyl Benzoate</td>
<td>Benzemul Application (Benzyl Benzoate 250mg/mL)</td>
<td>J. McGloin (Castle Hill, NSW)</td>
</tr>
<tr>
<td>Permethrin</td>
<td>Lyclear (5% w/w Permethrin)</td>
<td>Pfizer (West Ryde, NSW)</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>Equimec™ (Ivermectin 10mg/mL) mixed with BP88 to final concentration 100µg/g</td>
<td>Merial (Paramatta, NSW)</td>
</tr>
<tr>
<td>Tea tree oil</td>
<td>Melaleuca Oil (100%) mixed with BP88 to final concentration 5%</td>
<td>Thursday Plantation (Ballina, NSW)</td>
</tr>
</tbody>
</table>

### Table 2.2: Example of data collection form used in in vitro sensitivity testing

<table>
<thead>
<tr>
<th>Label</th>
<th>Due Time</th>
<th>Life stage</th>
<th>0</th>
<th>15m</th>
<th>30m</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Walking</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stored</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paralysis</td>
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<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>Dead</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOD</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Missing</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
2.4 Molecular Methods

2.4.1 Preparation of S. scabiei var. hominis genomic DNA

Mites were homogenised in either 20 or 50 µL of PrepMan Ultra™ solution (Applied Biosystems, Foster City, CA, USA) using a motorized micropestle (Kontes). Samples were incubated at 95°C for 10 minutes then cooled on ice prior to storage at -20°C. For mites stored originally stored in digestion buffer (500 µg/mL proteinase K, 50mM Tris-HCl, 1mM EDTA, 0.5% SDS, pH 8.5), 20 µL sterile distilled water was added and the samples incubated at room temperature for 2 hours prior to use.

2.4.2 Total RNA extraction

Live S. scabiei mites (10-50) were homogenised vigorously in 50-100 µL cold TRIzol™ reagent (Invitrogen, Mount Waverley, VIC, Australia), and stored at -80°C until use. Sterile RNAse free plasticware was used for all procedures and all steps were performed on ice with centrifugation at 4°C. Samples were thawed and homogenised again before use. After adding 400 µL TRIzol and 100 µL chloroform the mixture was agitated and incubated at room temperature for 3 minutes. The sample was centrifuged and the aqueous phase transferred to a chilled eppendorf tube. 250 µL isopropanol was added and the sample incubated for on ice for 2 hours prior to centrifugation. The pellet was washed in 1 mL 75% ethanol, air-dried briefly and resuspended in 10 µL RNAse free dH2O. Finally, the RNA was denatured at 65°C for 10 minutes, cooled on ice, and stored at -20°C. For use in qRT-PCR, RNA samples were additionally treated with 10U DNasel according to the manufacturer’s protocol (Invitrogen).

2.4.3 Reverse transcription

All steps were performed on ice. 5-6 µL RNA was mixed with ice cold RNase free dH2O to final volume of 12 µL. If not proceeding directly from RNA extractions and DNase treatment, samples were denatured at 65°C for 10 minutes and rapidly cooled on ice. Reactions contained 1 X RT buffer, 0.5 mM dNTPs, 1µM random nonamers (Promega), 10U RNase inhibitor (Invitrogen) and 1 µL Sensiscript or Omniscript (Qiagen) RT in 20 µL reaction volume. Reactions were incubated at 37°C for 1.5 hours, followed by 70°C for 10 minutes to inactivate the reverse transcriptase.
2.4.4 PCR

All PCR primers were synthesised by Sigma-Proligo (Lismore, NSW, Australia). Primer sequences and PCR conditions are described in the text. In general, PCR reactions contained 1 x PCR buffer, 0.2mM dNTPs (Roche), 0.4µM primers and 0.2U Taq DNA polymerase (Qiagen, Doncaster, VIC, Australia). For PCR of mite DNA prepared in digestion buffer containing SDS (2.4.1), 2% (v/v) Tween-20 was added to prevent PCR inhibition. Reactions contained 1-2 µL template DNA in a final volume of 25µL. PCR products were visualised on 1-2% (w/v) TAE agarose gels, stained with 0.5µg/mL Ethidium Bromide, and viewed on a UV transilluminator.

2.4.5 Contig extension PCR

A semi-nested PCR approach was commonly applied to the bacteriophage libraries in an attempt to extend existing contig sequences towards the 5’ end. This combined vector and nested gene specific primers. In general, the bacteriophage T3 primer was combined with sequence specific reverse primers. First round PCR products were diluted 10-fold and 100-fold, and subjected to a second round of PCR using the sequence specific nested primer. Products of interest were purified, cloned, sequenced, and aligned with existing contigs.

2.4.6 Measurement of DNA concentration

Estimates of DNA concentration and quality were made by running small aliquots on agarose gels in comparison to the GeneRuler 100bp ladder™ (MBI Fermentas, Paddington, NSW, Australia), which allowed accurate quantification of DNA between 8 and 80ng. Plasmid DNA was generally diluted to be in this range. Alternatively, the GeneQuant Pro™ spectrophotometer was used to determine DNA concentration and purity using A260/280 ratios using an ultramicrovolume cuvette (7µL capacity) according to the manufacturers instructions.

2.4.7 Cloning of PCR products

PCR products were purified by gel extraction or directly from PCR using QiaPrep or MinElute purification kits (Qiagen). The concentration and quality of products were determined before proceeding with ligations. The pGEM-T Easy vector system (Promega, Annandale, NSW, Australia) was used for most cloning applications.
Ligations were performed according to the manufacturers protocol, containing 1 x ligation buffer, 50ng vector, 1-3 μL PCR product, 3U T4 DNA ligase and dH2O to final volume of 10 μL. Ligations were incubated at 4°C overnight.

*Escherichia coli* strains DH5α or XL1-Blue were used for transformations. To prepare competent cells, a 10 mL starter culture was used to inoculate 200 mL LB (10% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl), and cells grown at 37°C with gentle shaking to an OD₆₀₀ of 0.5-0.6. Cells were briefly incubated on ice, prior to centrifugation at 4000 x g for 30 minutes at 4°C. The pellets were washed in 40 mL ice-cold dH₂O and centrifuged for 25 minutes. This step was repeated, then cells resuspended in 5 mL ice-cold 10% (v/v) glycerol prior to final centrifugation. Finally, cell pellets were resuspended in 270 μL 10% glycerol, and stored in 40 μL aliquots at -80°C until ready for use.

2 μL of the ligation reactions were mixed with competent cells and transformed using electroporation. Cells were quickly resuspended in 900 μL SOC media (2g Bacto-tryptone, 0.5% w/v Bacto-yeast, 10mM NaCl, 2.5mM KCl, 10mM Mg²⁺, 20mM glucose) and resuscitated for 30-60 minutes at 37°C. Cells were plated onto LB agar supplemented with 50μg/mL ampicillin, 0.5mM IPTG and 80μg/mL X-Gal and incubated at 37°C overnight.

To screen recombinants, white colonies were picked using a sterile toothpick, agitated in 50 μL dH₂O and subcultured onto a reference plate. The water stock was incubated at 95°C for 15 minutes, cooled on ice, and centrifuged for 3 minutes. 1-2 μL of the supernatant was used as template for PCR using either M13 vector or insert specific primers.

### 2.4.8 Sequencing

Plasmid DNA was purified using either BioRad or Qiagen Miniprep kits according to the manufacturers’ protocol. PCR products were purified using Qiagen purification kits. All samples were quantified prior to sequencing. Sequencing reactions were performed by multiple sequencing facilities over the course of the project due to changes in institutional service provision guidelines. These included the Advanced Analytical Centre (James Cook University, Townsville, QLD), Newcastle DNA (Biomolecular Research Facility, Newcastle, NSW) and Bioscience North Australia
(Charles Darwin University, Darwin, NT). Chromatograms were viewed and edited using ChromasPro software (www.technelysium.com.au/ChromasPro.html). Contigs were assembled using DNAStar (Lasergene software), BioEdit (Hall, 1999) or ChromasPro software. Further sequence analysis is described in text.

2.4.9 Rapid Amplification of cDNA Ends (RACE)

Total RNA was extracted from 50 *S. scabiei* var. *hominis* mites and RACE performed using the BD SMART™ RACE kit. This kit is designed to enrich for 5’ ends, using a specially designed RNA oligo (SMART II A) that hybridises preferentially to the dC-tail added to the end of the completed RNA template in first strand synthesis. For 3’ RACE first strand synthesis, 2 µL total RNA (<100ng) was mixed with 1.2µM of 3’ CDS primer-A (modified oligo dT) and 1µL dH2O. This mixture was incubated at 70°C for 2 minutes and cooled on ice for 2 minutes. 1X first strand buffer, 2mM DTT, 1mM dNTPs and 1µL of Powerscript™ reverse transcriptase was added and the reaction incubated at 42°C for 1.5 hours. The reaction was diluted with 20 µL Tricine-EDTA buffer and incubated at 72°C for 7 minutes prior to storage at -20°C. For preparation of 5’ RACE ready cDNA, 3 µL RNA was mixed with 1.2µM 5’ CDS primer (modified lock-docking oligo-dT) and 1.2µM BD SMART II A oligo.

2.5 Library screening

2.5.1 Source of *S. scabiei* var. *hominis* bacteriophage libraries

cDNA libraries were prepared by Dr. Katja Fischer from the Queensland Institute of Medical Research. Approximately 500 mites collected from a crusted scabies patient (prior to ivermectin treatment) were used to construct Oligo-dT primed cDNA. cDNA was directionally cloned into the λZAP express system (Stratagene, La Jolla, CA, USA) using the restriction enzymes EcoRI and XhoI. A database of 9216 expressed sequence tags (ESTs) was constructed from prenormalised sub libraries Yv4, 5 and 6; and are described further in Fischer *et al.* (2003a). A long PCR and cDNA reassociation procedure to normalise the library and remove the most abundant transcripts (described in Fischer *et al.*, 2003b) resulted in a further 34560 ESTs, with libraries referred to as Yv7, Yv8 and Yv9. Additionally, an *S. scabiei* var.
hominis genomic DNA library (SSNY#1) was prepared via BamHI cloning into the λZAP system by Dr. Deborah Holt.

2.5.2 Hybridisation based library screening

Plating and titring of bacteriophage libraries was performed as described in the λZAP express manual. The phage titer was determined by the following formula:

\[
\text{pfu/mL} = \frac{\text{Number of plaques} \times \text{dilution factor} \times \text{1000}}{\text{volume plated (µL)}}
\]

For primary screening of Yv7, 20 plates were prepared at a density of approximately 50,000 pfu per plate. Plates were incubated at 37°C for approximately 9 hours, and stored overnight at 4°C. Plaque lifts were performed using either HybondC or HybondXL filters (Amersham, Castle Hill, NSW, Australia). Filters were left to transfer for 2 minutes, denatured (1.5M NaCl, 0.5M NaOH) 2 minutes, neutralised (1M TrisCl, 1.5M NaCl, pH 7.4) 5 minutes, and rinsed in 2 x SSC (20 x SSC contains 3M NaCl, 0.3M Tri-Sodium Citrate), 0.2M Tris, pH 7.5. Filters were air-dried on blotting paper and fixed either by baking at 80°C for two hours or by UV cross-linking for 2 minutes.

To generate probes, PCR products were purified and labeled with $\alpha^{32}$P dCTP using the Redivue random priming labeling kit according to the manufacturers protocol (Amersham). Excess labeled nucleotides were removed using S200HR columns (Amersham). Filters were prehybridised for 2 hours at 55-65°C in 6 x SSC, 5 x Denhardts (0.5g Ficoll, 0.5g polyvinylpyrrolidone, 0.5g BSA per 50mL), 0.5% w/v SDS and 0.1mg/mL denatured herring sperm DNA. The denatured probe was added to this and hybridizations were performed overnight at 55-65°C

Following hybridization, filters were washed three times at 55-65°C in 2 x SSC, 0.1% w/v SDS. Membranes were sealed in plastic wrap, exposed to X-Ray film (X-OMAT AR, Kodak) at -80°C for 1-2 days and developed.

Films showing positive plaques were aligned with original plates and plaques cored from the agar using a sterile Pasteur pipette. Phage plugs were placed into 1 mL SM buffer (2.5g NaCl, 1g MgSO$_4$, 25mL 1M Tris, 2.5mL 2% w/v gelatin per 500mL)
and 20µL chloroform, vortexed, and stored at 4°C. For secondary screening, first round phage stocks were diluted 1:100 in SM buffer and 10 µL used to infect XL1-Blue cells (about 1000pfu/plate). Secondary plaque lifts and hybridization conditions were performed in an identical manner. Selected positive plaques were excised according to the λZAP express manual. To plate excised phagemids, 10 µL of phage supernatant was mixed with 200 µL XLOLR cells (OD_{600} 1.0) and incubated for 15 minutes at 37°C. 3 mL NZY broth (5g NaCl, 2g MgSO_4, 5g yeast extract, 10g NZ amine per litre) was added and incubation continued for 45 minutes. 200 µL of the cell suspension was plated onto LB/Kanamycin plates and incubated overnight at 37°C.

### 2.5.3 PCR based library screening

A high-stringency PCR based screening approach was employed, based on the method described by Israel (1993). This method allows the relatively simple isolation of complete phagemid clones; provided that sufficient sequence information exists, and minimizes the use of radioactivity. Library titration and dilution series PCRs determined the minimal starting amount of library phage DNA required to generate a PCR product. This required amount of phage was used in first round of screening to infect 1 mL *E. coli* XL1-Blue MRF’ cells at OD_{590} 1.0. 18 mL LB broth was added and the mixture plated in an 8 X 8 matrix (100 µL per well) in a 96-well plate. The plate was sealed and incubated at 37°C with shaking for five hours. Rows and columns were pooled by taking a 20 µL aliquot from each well and diluting 1:1 with distilled water. 10 µL chloroform was added to the pooled phage suspensions and reactions were centrifuged briefly prior to PCR. Single PCR positive wells were selected and the above process repeated several times to enrich the number of positive clones for each round of screening.

After multiple rounds of screening, approximately 2000 pfu from the PCR positive well were plated and plaque lifts and hybridizations performed as described above.

Hybridisation and PCR positive plaques were cored from the agar and phagemid excision was performed according to the λZAP express protocol.
Chapter 3

Analysis of *Sarcoptes scabiei* var. *hominis* in vitro sensitivity to ivermectin, 1997-2006

3.1 Introduction

Assessment of drug efficacy in scabies is currently largely based on clinical reports. This can be problematic due to the difficulties in diagnosis and the low numbers of mites present in ordinary scabies. Clinically, it can be difficult to differentiate between an active infestation, re-infestation, or residual skin reaction after treatment. These factors, coupled with the lack of diagnostic test, availability of animal model, or culture system for scabies mites, mean that the capability to conduct in vitro drug sensitivity assays involving *S. scabiei* is generally limited.

We have overcome many of these problems by utilising the shed skin of crusted scabies patients to obtain sufficient numbers of mites for analysis. Our laboratory has previously reported the employment of a simple in vitro assay to measure relative acaricide efficacy in crusted scabies patients presenting to Royal Darwin Hospital (Walton *et al.*, 2004c; Walton *et al.*, 2000). Survival data has now been collected for over ten years and is a valuable source of information regarding the efficacy of scabies treatment in northern Australia. A recent study highlighted the acaricidal properties of tea tree oil, with this agent now commonly employed in the treatment of crusted scabies (Walton *et al.*, 2004c). Previous in vitro studies have also demonstrated that *S. scabiei* var. *hominis* were becoming increasingly tolerant to permethrin, raising concerns about the long term sustainability of current mass community intervention programs utilising permethrin (Walton *et al.*, 2000).

Oral ivermectin has been proposed as a possible alternative acaricide for mass treatment programs. Although not formally licensed for scabies treatment in Australia, it was first approved for compassionate use in April 1992 (Currie *et al.*, 1994), and multiple dose regimens have been increasingly used since 1996 for the management of crusted scabies in northern Australia. However reported clinical and
in vitro resistance (Currie et al., 2004) suggested that careful monitoring and vigilance is required to prevent the emergence of drug resistance. Collection of longitudinal data has allowed us to extend previous analysis to determine whether ivermectin resistance or tolerance was isolated to specific years, patients, or treatment regimens, and if trends are emerging over time.

3.2 Methods

Details on in vitro assay methodology are presented in chapter 2. Analysis was based on survival curve analysis using 100µg/g ivermectin (the most commonly used concentration). Log-rank tests were applied to test for statistical significance. Also known as the Mantel-Haenszel test, this tests the null hypothesis that survival curves are identical in overall populations. All database entries were cross-referenced to hard-copies of sensitivity data sheets (Chapter 2, Table 2.2) to ensure data validity.

3.3 Results

Over ten years, an average of 22 patients per annum were admitted to Royal Darwin Hospital with crusted scabies, although mite numbers were only sufficient to collect ivermectin sensitivity data from 16 individual patients, some on multiple occasions. Sensitivity assays were conducted on average three times per year, and a total of 514 mites were assayed with ivermectin (Table 3.1). From 1997-1999, mites were uniformly sensitive to ivermectin, with a median in vitro survival time of 60 minutes. Median survival times increased significantly to 210 minutes in 2000, and have been relatively stable in recent years, with a median of 120 minutes over the last three years (Table 3.1). In comparison, over the ten years of collection, median survival times remained unchanged for the negative control ointment BP88 (681 minutes), and 20 minutes with the positive control acaricide benzyl benzoate (data not shown).

To investigate trends in more detail, sensitivity data from two recurrent crusted scabies patients with previously documented ivermectin resistance (Currie et al., 2004) were compared. Mites from both patients were highly sensitive to ivermectin in the first three years of monitoring, with a median survival time of 60 minutes.
Significantly increased survival times were observed in 2000 in both patients, but were most dramatic in patient 1 (Figure 3.1, 3.2, Table 3.2, 3.3). Between the years 2001-2006, survival times of mites isolated from patient 2 were largely consistent (Figure 3.2, Table 3.3). Mites from patient 1 however returned to the previous highly-sensitive levels in 2001 and 2002, before increasing tolerance was observed again in 2003 (Figure 3.1).

Table 3.1: Aggregate ivermectin survival times, all patients, 1997-2006

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of patients tested</th>
<th>No. of mites assayed with ivermectin</th>
<th>Median survival time (min)</th>
<th>P (logrank test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td>5</td>
<td>20</td>
<td>60</td>
<td>0.0005</td>
</tr>
<tr>
<td>1998</td>
<td>3</td>
<td>20</td>
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<td>0.0005</td>
</tr>
<tr>
<td>1999</td>
<td>2</td>
<td>11</td>
<td>60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2000</td>
<td>4</td>
<td>209</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>4</td>
<td>58</td>
<td>120</td>
<td>ns^a</td>
</tr>
<tr>
<td>2002</td>
<td>3</td>
<td>27</td>
<td>145</td>
<td>ns</td>
</tr>
<tr>
<td>2003</td>
<td>2</td>
<td>12</td>
<td>210</td>
<td>ns (0.16)</td>
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<tr>
<td>2004</td>
<td>2</td>
<td>35</td>
<td>120</td>
<td>ns</td>
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<tr>
<td>2005</td>
<td>1</td>
<td>41</td>
<td>120</td>
<td>ns</td>
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<tr>
<td>2006</td>
<td>5</td>
<td>81</td>
<td>120</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>31</strong></td>
<td><strong>514</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a ns = not significant

Sensitivity data obtained from all other crusted scabies patients was also examined. Similar trends were observed, with mites highly sensitive to ivermectin in 1997 and 1998, followed by a decreased sensitivity in 2000. In 2004 mites isolated from one patient showed apparent in vitro resistance, with 40% of mites still alive after overnight exposure (Figure 3.3, Table 3.4). Of considerable interest was the recent admission of CS patient 2, in which sensitivity assays were performed over a time course of ivermectin treatment. Of note, this patient has now received approximately 130 doses of ivermectin in total since 1996 (B. Currie, pers. comm.) Mites were collected from CS patient 2 and assays performed on days 0, 3, 6 & 8 of admission, with ivermectin administered on days 0, 1 & 7. The first three assays indicated mites were highly sensitive to ivermectin (median survival = 60 minutes). By day eight however, survival time had tripled (180 minutes, p = 0.0047) (Figure 3.4).
Figure 3.1: Kaplan-Meier survival analysis of mites exposed to ivermectin, collected from recurrent crusted scabies patient 1. Significance levels: * = <0.05, ** = <0.01, *** = <0.0001

Table 3.2: Median mite survival times to ivermectin from recurrent crusted scabies patient 1.

<table>
<thead>
<tr>
<th>Year</th>
<th>Ivermectin median survival time (min)</th>
<th>P (log rank test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>60</td>
<td>ns</td>
</tr>
<tr>
<td>2000</td>
<td>360</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>2001</td>
<td>60</td>
<td>ns</td>
</tr>
<tr>
<td>2002</td>
<td>97</td>
<td>ns</td>
</tr>
<tr>
<td>2003</td>
<td>270</td>
<td>0.04*</td>
</tr>
</tbody>
</table>
Figure 3.2: Kaplan-Meier survival analysis of mites exposed to ivermectin, collected from recurrent crusted scabies patient 2. Symbols are described in figure 3.1.

Table 3.3: Median mite survival times to ivermectin from recurrent crusted scabies patient 2.

<table>
<thead>
<tr>
<th>Year</th>
<th>Ivermectin median survival time (min)</th>
<th>P (log rank test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td>60</td>
<td>0.001**</td>
</tr>
<tr>
<td>1998</td>
<td>180</td>
<td>ns</td>
</tr>
<tr>
<td>2000</td>
<td>204</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>2001</td>
<td>120</td>
<td>ns</td>
</tr>
<tr>
<td>2004</td>
<td>120</td>
<td>ns</td>
</tr>
<tr>
<td>2005</td>
<td>120</td>
<td>ns</td>
</tr>
<tr>
<td>2006</td>
<td>120</td>
<td>ns</td>
</tr>
</tbody>
</table>


**Figure 3.3:** Kaplan-Meier ivermectin survival analysis of mites obtained from all other crusted scabies patients (excluding patients 1 & 2).

**Table 3.4:** Median mite survival times to ivermectin from all other crusted scabies patients.

<table>
<thead>
<tr>
<th>Year</th>
<th>Ivermectin median survival time (min)</th>
<th>P (log rank test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td>20</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>1998</td>
<td>20</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>2000</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>161.5</td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>335</td>
<td>&lt;0.03*</td>
</tr>
<tr>
<td>2006</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4: Sensitivity of mites collected from CS patient 2 over a time course of ivermectin treatment.

3.4 Discussion

Analysis of sensitivity data clearly show that *S. scabiei* have developed increased tolerance to ivermectin since its introduction as an acaricide for the management of crusted scabies in northern Australia. Median survival times in vitro have more than doubled over the ten year period investigated. This increase was first observed in 2000, corresponding to the first reports of clinical ivermectin failure in two patients with recurrent crusted scabies (Currie et al., 2004). Over the last six years, survival times have remained largely consistent; however they have not returned to the more sensitive levels seen prior to 2000.

In addition to this trend of increasing ivermectin tolerance, two further cases of significantly increased ivermectin survival were seen. The first occurred in 2003, in one of the patients with previously documented resistance (Table 3.2); the second, in 2004, from a third recurrent crusted scabies patient who has also received multiple doses of ivermectin over a period of several years (Table 3.4). Prior to admission to Royal Darwin Hospital, this patient had received a single dose of ivermectin with little clinical effect. Subsequently however, an excellent response was observed with
CHAPTER 3

the combination of ivermectin with benzyl benzoate/tea-tree oil topical therapy (Currie, pers. comm.). No further sensitivity data has been collected from these patients since these admissions, therefore we are unable to establish whether these observations were isolated (as observed in 2000), or if tolerance to ivermectin is building in mites from these patients.

There are a number of limitations to the in vitro assays described herein. Although assays are standardized as much as possible, there are still areas of subjectivity. The main problem is that the assays are labour intensive and time consuming. This means that only a limited number of mites can be tested per assay to maintain accuracy. Often mites cannot be monitored regularly as desired, particularly when assays continue overnight, and occasionally time of death cannot be determined precisely. When this occurs data is interpreted as the last time point seen alive rather than observation of death, therefore results may be an under representation of actual survival.

An ideal bioassay should be robust, simple and reproducible. Most importantly, the tests should be sensitive enough to detect differential drug responses applicable to the clinical or field setting (Denholm et al., 2002). Although these “maximum exposure” survival assays fulfil many of these criteria, whether they are adequately sensitive to detect subtle, but clinically relevant changes is questionable, particularly in the case of ivermectin. While the other topical acaricides tested are over-the-counter products and concentrations, ivermectin is diluted to 100µg/g. At what level of sensitivity this can distinguish between resistant and sensitive mites is uncertain. Ivermectin is thought to be delivered to the mite primarily by digestion of sera and epidermal cells (Burkhart, 1999), so whether the in-vitro assay delivery method is comparable to this could be disputed. This is complicated by the fact that there is little information regarding the acaricidal concentrations of ivermectin in the skin, and how well the concentrations used in these assays correlate to this.

Many other arthropod bioassays use LC$_{50}$ mortality assessment rather than survival or knockdown assays. An LC$_{50}$ test would potentially provide a great deal more sensitivity, detecting more subtle changes in mite tolerance. They may also be less time consuming as survival times do not need to be monitored as frequently, and assays can be conducted over a shorter time frame. To establish accurate LC$_{50}$
values, larger mite numbers may be required, which would be difficult to achieve in the clinical arena. For example in spider mites Kabir et al. (1996) recommends a minimum of five concentrations and 480 mites be tested for reliable LC\textsubscript{50} estimates, although a three-concentration design using 240 mites may also be acceptable. Furthermore, because multiple treatments are tested in any given assay, LC\textsubscript{50} assays would need to be developed and performed for each acaricide. These alternate assay methodologies are currently being developed in the Menzies School of Health Research laboratories, but are limited by access to large numbers of mites.

Alternative application methods such as solutions or acaricide impregnated filter paper may enhance assay reproducibility and would also save time, as our method of manually coating plates is labour intensive and could introduce variability to the assay. However in our experience, mites actively wander on plates, are often found on the sides and lids of Petri dishes, and may preferentially move to these sites in avoidance of the acaricide coated filter paper. Importantly, the plate coating method has given comparable results in other laboratories, attesting to the reproducibility of the assay (Arlian, unpublished). Furthermore, one may argue that the use of over-the-counter topical ointments is much more relevant clinically, and with our use of consistent methodology over the period investigated, we can now define a “normal” in vitro response to acaricides with some confidence.

Despite assay limitations, from ten years of data it appears that changing treatment strategies have impacted mite sensitivity in vitro. Between 1995 and 2000 treatment for crusted scabies largely involved the application of 5% permethrin and the increasing use of single dose ivermectin. It quickly became apparent that multiple doses of ivermectin were required, with early treatment failures reported (Currie et al., 1995; Huffam and Currie, 1998). Molecular genotyping found that in severe cases of crusted scabies, even three doses of ivermectin within two weeks was insufficient to prevent relapse (Walton et al., 1999b). Due to these inadequate responses, in recent years there has been an increased focus on combining topical and systemic therapy. For the most severe cases ivermectin is now administered at Royal Darwin Hospital as a seven dose course on days 0, 1, 7, 8, 14, 21 and 28. Additionally 25% benzyl benzoate with 5% tea-tree oil is administered on alternate days with keratolytic cream (Royal Darwin Hospital, 2006a). In vitro, benzyl benzoate and tea tree oil are extremely efficacious, with median survival rates 20 and
60 minutes respectively (this chapter, Walton et al., 2004c). These combination treatment regimens have been very successful, and after a week of therapy live mites are rarely observed, with ‘cures’ normally obtained within two weeks. Re-infection remains a problem however, either by reinfestation from untreated contacts, or recrudescence due to incomplete eradication of eggs. Since the adoption of these intensive treatment protocols, survival curves have remained extremely consistent, attesting to the usefulness of combination therapy.

The clinical response of patients to drug treatment is determined by many factors, not only mite drug sensitivity phenotype. In regard to ivermectin, efficacy may be influenced by the dose, absorption of the drug, distribution to tissues, and importantly, the co-administration of other drugs. For this reason, we find that clinical and in vitro data occasionally differs. For example, the observations of in vitro resistance in 2004 (Figure 3.3) were not correlated with a poor clinical response. The use of a combination therapy makes it very difficult to determine the actual impact of multi-dose ivermectin, as benzyl benzoate treatment may mask true ivermectin efficacy.

The cases of clinical resistance in 2000, and reports of poor responses to ivermectin therapy alone indicate that when ivermectin is used in the absence of benzyl benzoate for crusted scabies, selection for drug resistant mites may occur rapidly. This is highlighted by a recent case, where due to compliance issues; the commencement of benzyl benzoate/tea tree oil therapy was delayed. The patient was severely infested and despite three doses of ivermectin no reduction in mite numbers were observed in skin scrapings. A significant increase in mite survival time was observed when mites collected after three doses were compared to those collected prior to the commencement of ivermectin therapy (Figure 3.4). It should be noted however that although this increase was observed, overall mite in vitro survival times remained within the “normal” ranges observed since 2004 (≤ 120 minutes).

Because most ordinary scabies patients have fewer than 10 mites, sensitivity assays are restricted to the more severe cases of crusted scabies. Furthermore, assays must be initiated within several hours of collection to circumvent differences in mite survival ability away from the host. Although mites may survive and remain infective for up to 36 hours at room conditions, penetration slows with increasing
time away from the host, concordant with the mites weakened status (Arlian et al., 1984a). Consequently, our assays are restricted to patients admitted to Royal Darwin Hospital to enable timely transport to the laboratory. As treatment for crusted scabies is now increasingly coordinated within regional centres via remote community clinics, access to patients is becoming more limited. However, surveillance of community crusted scabies patients is extremely important, not only to the patient but to the community as a whole. Crusted scabies patients have been identified as core transmitters in many communities, and may explain the limited sustainability of community control programs (Currie et al., 1994). Factors such as unsupervised, suboptimal therapy and limited follow up may facilitate the spread of ivermectin resistance. Consequently, the transmission of ivermectin resistant mites from crusted scabies patients to others in the community would seriously threaten the success of any future mass treatment strategy involving ivermectin. If we are to maintain insights into the potential development of ivermectin resistance, there is an urgent need for increased monitoring within the community setting, as seen in veterinary programs.

With ivermectin tolerant mites now observed in three crusted scabies patients on four separate occasions, there is a need to identify genes under ivermectin selection and the molecular basis for drug resistance. This work will become increasingly important if ivermectin is indeed incorporated into mass treatment programs. In the meantime, the continuation of in vitro testing remains an important adjunct to routine clinical practice for individual patients and recent community initiatives to ensure the successful treatment of scabies. It was on this basis that the following investigations were initiated.
Chapter 4

Identification of ABC transporter genes from *Sarcoptes scabiei*

4.1 Introduction

A potential mechanism for ivermectin resistance is increased cellular export mediated by ATP-binding cassette (ABC) transporters. ABC transporters can confer multidrug resistance by transporting a broad range of substrates across membranes, thus leading to decreased intracellular accumulation. ABC transporters are presently grouped into eight subfamilies according to sequence similarity and domain organization (Sheps *et al.*, 2004), (section 1.13.1). Several of these proteins have been associated with drug resistance, the most well-known of which is the ABC-B transporter P-glycoprotein.

P-glycoprotein was first discovered to be over-expressed in multidrug resistant cancer cells; hence the common referral of P-glycoprotein as the multidrug resistant gene (*mdr*). Increased expression of P-glycoprotein in these cells confers resistance to a wide range of hydrophobic drugs (reviewed by Gottesman *et al.*, 1995). Ivermectin is known to be an excellent substrate for ABC transporters such as P-glycoprotein (Nobmann *et al.*, 2001), with mammals deficient in P-glycoprotein displaying hypersensitivity to ivermectin (Roulet *et al.*, 2003; Schinkel *et al.*, 1994). Several molecular studies suggest the involvement of P-glycoprotein in ivermectin resistance, although a functional association has not yet been demonstrated. Early research found alterations to P-glycoprotein and increased mRNA levels in ivermectin resistant *Haemonchus contortus* (Xu *et al.*, 1998). Selection at a P-glycoprotein allele following ivermectin treatment has been observed in *H. contortus* (Blackhall *et al.*, 1998a), and more recently *Onchocerca volvulus* (Ardelli *et al.*, 2005a; Eng and Prichard, 2005).

It is possible that P-glycoproteins or other ABC transporters might play an important role in the development of ivermectin resistance in *Sarcoptes scabiei*. To investigate mechanisms of ivermectin resistance, an important preliminary step is to identify and
characterise candidate resistance genes. In this study, an EST library and database was utilised to identify potentially relevant ABC transporter genes from *S. scabiei*.

### 4.2 Methods

#### 4.2.1 Searching the *S. scabiei* var. *hominis* EST database

Putative ABC transporters were initially identified by comparing the *S. scabiei* dataset of 43,776 ESTs (section 2.5.1) to GenBank using BLASTx (Altschul *et al.*, 1990). Additional putative ABC transporters were identified by searching the EST dataset for homologues of the P-glycoprotein ABC transporters, Pgp-49 (Q00449) from *D. melanogaster*, and Pgp-A (AAC38987) from *H. contortus*, using the full length cDNAs as the query sequence. Contigs identified as potential ABC transporters were further analysed using BLASTp.

#### 4.2.2 Sequence extension of EST contigs

Six EST contigs were initially selected for further sequencing. Additionally, several cDNA clone sequences were incomplete and internal primers were designed to complete the insert sequence. To extend the 5’ ends of existing sequence, a semi-nested PCR approach was taken as described in section 2.4.5. In the case of contig 7008C03, the 3’ end of the putative protein was also incomplete, so nested forward primers were combined with the vector T7 primer (Table 4.1). PCR reaction components are described in chapter 2. Reactions were cycled at 94\(^{\circ}\)C for 30 seconds, 56\(^{\circ}\)C for 30 seconds and 72\(^{\circ}\)C for 1 minute and 30 seconds for 35 cycles, followed by a final extension step of 72\(^{\circ}\)C for 10 minutes (annealing times varied according to primer Tm). PCR products were purified, cloned and sequenced with M13 primers as described in chapter 2.
### Table 4.1: Primers used for extension and sequencing of EST contigs

<table>
<thead>
<tr>
<th>Contig</th>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
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</thead>
<tbody>
<tr>
<td>4012G02</td>
<td>G02F1</td>
<td>CGT CTC ATT GAC TTA TAT CTG G</td>
</tr>
<tr>
<td></td>
<td>G02R1</td>
<td>TTC GAG ATC AAT AGC CGT A</td>
</tr>
<tr>
<td></td>
<td>G02R3</td>
<td>CTA AAA TTC GAG TGC GAT CT</td>
</tr>
<tr>
<td></td>
<td>G02R4</td>
<td>TTA GCG ACA AGA TCA AC</td>
</tr>
<tr>
<td>8060B04</td>
<td>B04R1</td>
<td>GTC TGC TAA CCA ACG ATT AGC</td>
</tr>
<tr>
<td></td>
<td>B04R2</td>
<td>GCT CTA ATA GTC GAT ACA CC</td>
</tr>
<tr>
<td></td>
<td>B04R3</td>
<td>ATC GCT AAA GCA CCG ATC AC</td>
</tr>
<tr>
<td></td>
<td>B04R4</td>
<td>ACT AAA ATT CGA GTG CGA TCT</td>
</tr>
<tr>
<td></td>
<td>B04R5</td>
<td>TTG GCG ACA TGA GAA TCA AC</td>
</tr>
<tr>
<td></td>
<td>B04R6</td>
<td>GTT GAA TGG ACT TCC GAA CA</td>
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<tr>
<td></td>
<td>B04R7</td>
<td>GTA ACA TTC TGA ATC CAG GC</td>
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<td>E12R1</td>
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<td>E12R2</td>
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<tr>
<td>7008C03</td>
<td>C03F1</td>
<td>TAG TAT TGC ACA TGC ACC GA</td>
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<td>C03F2</td>
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<tr>
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<td>CCA ACA TCT TCA ATG TGT CC</td>
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<td>CGC CAG GGT TTT CCC AGT CAC GAC</td>
</tr>
<tr>
<td></td>
<td>M13r</td>
<td>TCA CAC AGG AAA CAG CTA TGA C</td>
</tr>
</tbody>
</table>
4.2.3 Identification of a P-glycoprotein sequence using degenerate PCR

To remove *E. coli* contamination, the cDNA libraries were treated with DNase I to digest non-phage DNA. A fresh aliquot of the pre-normalised (Yv4) and normalised (Yv7) cDNA library was mixed with 10U DNase I (Roche, Castle Hill, NSW, Australia) and 2.5mM of MgCl₂ and incubated at 37°C for two hours. The reaction was heat inactivated for 10 minutes, cooled and purified using the QiaQuick reaction clean up kit (Qiagen).

Degenerate primers for amplifying P-glycoprotein cDNA fragments were employed based on the highly conserved ATP-binding domains of P-glycoprotein. This approach has been successfully used to isolate P-glycoprotein in several other studies (Huang and Prichard, 1999; Kwa et al., 1998; Sangster et al., 1999; Xu et al., 1998). *S. scabiei* var. *hominis* cDNA libraries Yv4 and Yv7 and multi-mite genomic DNA preparations were used as templates for nested PCR. First round sense and antisense 1 primers (Table 4.2) were used in 16 possible combinations. Cycling conditions were 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute and 30 seconds, for 35 cycles followed by a final extension step of 72°C for 10 minutes. 1 µL of the first round PCR products were used as a template for the second round reaction with antisense 2 primers (Table 4.2) with the above parameters employed.

**Table 4.2: Degenerate PCR primers based on ATP-binding domain of P-glycoprotein**

<table>
<thead>
<tr>
<th>Direction</th>
<th>Peptide Sequence</th>
<th>Name</th>
<th>Primer sequence* (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>SGCGKST</td>
<td>mdrF1</td>
<td>TCD GGI TGY GGN AAR TCD AC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mdrF2</td>
<td>AGY GGI TGY GGN AAR TCD AC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mdrF3</td>
<td>TCD GGI TGY GGN AAR AGY AC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mdrF4</td>
<td>AGY GGI TGY GGN AAR AGY AC</td>
</tr>
<tr>
<td>Antisense 1</td>
<td>DEATSALD</td>
<td>mdrR1</td>
<td>TCI AGI GCI GAN GTN GCY TCR TC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mdrR2</td>
<td>TCI AGI GCR CTN GTN GCY TCR TC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mdrR3</td>
<td>TCY AAI GCI GAN GTN GCY TCR TC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mdrR4</td>
<td>TCY AAI GCR CTN GTN GCY TCR TC</td>
</tr>
<tr>
<td>Antisense 2</td>
<td>GQKQRIAI</td>
<td>mdrR5</td>
<td>ATI GCD ATI CGY TGY TTY TGN CC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mdrR6</td>
<td>ATI GCD ATY CTY TGY TTY TGN CC</td>
</tr>
</tbody>
</table>

*Degenerate base abbreviations: D=G/A/T, Y=C/T, R=A/G, N=A/T/C/G, I= inosine
4.2.4 PCR based library screening for P-glycoprotein

The conserved region obtained through degenerate PCR was used to probe the normalised cDNA library to isolate the entire clone and thus a larger fragment of the gene. Primers corresponding to the sequence were designed to generate a 280bp PCR product (236F1/R1, Table 4.1). A high-stringency PCR based screening approach was employed (chapter 2).

After the 4th round of PCR library screening, plaque lifts were performed and 280bp $\alpha^{32}$P dCTP labeled probe was generated using the 236F1/R1 primers. Hybridisations were performed, and positive plaques excised (chapter 2). Plasmid DNA was extracted and inserts sequenced with the bacteriophage T3 & T7 primers (Table 4.1).

4.2.5 Sequence analysis

Edited DNA sequences were translated into amino acid sequences using the ‘Flip6 frames program’ (Brossard, 1997), accessed via Biomanager (www.angis.org.au). Amino acid sequences were submitted to BLASTp and conserved domain databases accessed via the NCBI server (http://www.ncbi.nlm.nih.gov/blast/). Putative transmembrane helices were detected in the peptide sequences using the program TM Pred (http://www.ch.embnet.org/software/TMPRED_form.html) (Hofmann and Stoffel, 1993). Sequences reported were submitted to the Genbank™ database and were assigned the accession numbers DQ146410-DQ146418.

4.2.6 Cluster analysis

Cluster analysis of the S. scabiei sequences was conducted to confirm the assignment of ABC subgroups, and to examine the relationship of the S. scabiei ABC transporters to sequences from C. elegans and D. melanogaster (Table 4.3). Peptide sequences corresponding to the conserved ATP-binding regions were aligned using ClustalW and bootstrapping for confidence determination was performed using Seqboot. Consensus phylogenetic trees were constructed using the Parsimony (Protpars) algorithm. Because the contig 7008C03 did not appear to contain an ATP-binding motif it was excluded from this analysis.
Table 4.3: Sequences used in cluster analysis of EST contigs

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Species</th>
<th>Protein</th>
<th>Accession</th>
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<tbody>
<tr>
<td>A</td>
<td><em>Caenorhabditis elegans</em></td>
<td>Y39D8C.1/Abt-4</td>
<td>AAC69223</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CG1718</td>
<td>AAF50837</td>
</tr>
<tr>
<td>B</td>
<td><em>Caenorhabditis elegans</em></td>
<td>K08E7.9/Pgp-1</td>
<td>CAB01232</td>
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<tr>
<td></td>
<td><em>Drosophila melanogaster</em></td>
<td>Mdr49</td>
<td>AAF58437</td>
</tr>
<tr>
<td>C</td>
<td><em>Caenorhabditis elegans</em></td>
<td>F57C12.5/Mrp-1</td>
<td>AAD31550</td>
</tr>
<tr>
<td></td>
<td><em>Drosophila melanogaster</em></td>
<td>CG6214</td>
<td>AAF53223</td>
</tr>
<tr>
<td>D</td>
<td><em>Caenorhabditis elegans</em></td>
<td>C54G10.3</td>
<td>CAA99810</td>
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<tr>
<td></td>
<td><em>Drosophila melanogaster</em></td>
<td>CG12703</td>
<td>AAF49018</td>
</tr>
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<td>E</td>
<td><em>Caenorhabditis elegans</em></td>
<td>Y39E4B.1</td>
<td>CAB54424</td>
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<tr>
<td></td>
<td><em>Drosophila melanogaster</em></td>
<td>CG5651</td>
<td>AAF50342</td>
</tr>
<tr>
<td>F</td>
<td><em>Caenorhabditis elegans</em></td>
<td>T27E9.7/GCN20-2</td>
<td>CAB04880</td>
</tr>
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<td></td>
<td><em>Drosophila melanogaster</em></td>
<td>CG9330</td>
<td>AAF49142</td>
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<td>G</td>
<td><em>Caenorhabditis elegans</em></td>
<td>C05D10.3</td>
<td>AAA20989</td>
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<td></td>
<td><em>Drosophila melanogaster</em></td>
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<td>AAF51548</td>
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<td><em>Caenorhabditis elegans</em></td>
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<td>AAA81093</td>
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<td></td>
<td><em>Drosophila melanogaster</em></td>
<td>CG9990-PA</td>
<td>AAF56807</td>
</tr>
</tbody>
</table>

4.3 Results

4.3.1 Identification and extension of EST contigs with similarity to ABC transporters

Preliminary analysis of a *S. scabiei* EST dataset of 43776 sequences, identified nine contigs with similarity to ABC transporters. Contig extension using a semi-nested PCR approach on the cDNA libraries and/or further sequencing of individual clones yielded additional sequence information. During this process, two of the previously separate contigs were found to overlap and were combined.
4.3.2 Sequence analysis of contigs

Four of the resulting eight *S. scabiei* contigs displayed significant identity to the multidrug resistance protein (MRP/ABC-C) family of ABC transporters (Table 4.4). 8060B04, 9002G04 and 7001E12 all aligned over the C-terminal region of their MRP homologues, while 7008C03 aligned over the central region of the protein, possibly between ATP-binding domains. This was supported by the absence of ATPase consensus regions in the 7008C03 sequence. Although the contigs presented here were homologous to similar proteins, alignments between the contigs indicated they were sufficiently different from each other not to be considered duplicate or different isoforms of a single *S. scabiei* protein.

Contig 7067D09 shared high levels of identity (78%) with the conserved RNAse L inhibitor proteins from many organisms. These proteins belong to the ABC-E subfamily of ABC transporters. Two ATP-binding domains were identified, and a conserved domain associated with the RNAase L inhibitor ATPase was also detected (Table 4.4).

The deduced amino acid sequence of the contig 7002E01 was determined to have significant similarity to the ABC-F transporter GCN20 from several organisms (Table 4.4). From alignment with the other proteins in this family, the sequence of 7002E01 was determined to be complete. Conserved domains of ATPase components were detected in two regions of the sequence. No transmembrane domains were identified in the protein, which is consistent with other ABC-F transporters.

Of the remaining two contigs, 4013B10 displayed significant similarity to the C-terminus of ABC-A proteins from various organisms (Table 4.4). The 7052B06 clone contig appeared to be chimeric, with BLASTp detecting no similarity over the first 150bp. The remainder of the protein aligned with the N-terminal ATP binding domain of ABC-H proteins from *Anopheles gambiae* and *D. melanogaster*, and ABC-G proteins from other organisms (Table 4.4).

As none of these contigs appeared to be members of the ABC transporter subfamily B, which includes the P-glycoproteins implicated in drug resistance in some
organisms, a further degenerate PCR approach was utilised in order to identify a member of this group.

### 4.3.3 Degenerate PCR

Semi-nested PCR of the DNase I treated Yv7 cDNA library using degenerate primers designed to the highly conserved ATP-binding domains of P-glycoproteins, successfully yielded products of the expected size for two primer combinations - mdrF2/R3/R5 and mdrF2/R3/R6. The fragment from the latter (designated mdr7-236) was cloned and sequenced. The sequence was found to share significant homology to P-glycoprotein from several organisms. No significant similarity was detected from BLASTn against the *E. coli* database indicating the sequence was not derived from *E. coli* contamination. Degenerate PCRs on genomic mite DNA preparations resulted in the cloning of two fragments. The first clone was amplified using the primer combination mdrF1/R4/R6. It was found to be identical to the mdr7-236 sequence but containing a 70bp intron. The second genomic clone was amplified with the primer combination mdrF3/R3/R5. Although no obvious introns were present, there were multiple stop codons and thus the sequence could not be translated to a single open reading frame. tBLASTx was performed on the nucleotide sequence, with the highest match being the N-terminal ATP-binding domain of CG3879/mdr 49 from *Drosophila melanogaster* (63% ID, 2e-12). The presence of multiple stop codons suggested the sequence might represent a pseudogene so it was excluded from further analysis.

### 4.3.4 PCR based library screening

The Yv7 cDNA library was enriched for mdr7-236 phage, through several rounds of PCR based screening and amplification. Hybridisation of the plated enriched stock with a mdr7-236 probe, yielded two positive plaques. These phages were confirmed to be positive via PCR with mdr7-236 specific primers then excised to phagemids and sequenced. The clones were found to contain a 1094bp insert which included the original mdr7-236 sequence. This extended mdr7-236 sequence showed homology to the C-terminal region of many P-glycoproteins, with the top BLASTp match being P-glycoprotein 3 from *Gallus gallus* (Table 4.4).
Table 4.4: *S. scabiei* ABC transporters identified in this study

<table>
<thead>
<tr>
<th>Contig</th>
<th>Length (aa)</th>
<th>% of total¹</th>
<th>BLASTp top match (Accession no.)</th>
<th>Description</th>
<th>No. ABC domains²</th>
<th>% id</th>
<th>e value</th>
<th>Sub family⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>8060B04</td>
<td>807</td>
<td>51</td>
<td><em>Danio rerio</em> (AAH56740.1)</td>
<td>MRP2 / ABCC2</td>
<td>2</td>
<td>50</td>
<td>0</td>
<td>C</td>
</tr>
<tr>
<td>9002G04</td>
<td>402</td>
<td>26</td>
<td><em>D. melanogaster</em> (AAS64699)</td>
<td>CG6214-PM (dMRP)</td>
<td>1</td>
<td>61</td>
<td>6e-125</td>
<td>C</td>
</tr>
<tr>
<td>7001E12</td>
<td>320</td>
<td>20</td>
<td><em>Apis mellifera</em> (XP395679)</td>
<td>Similar to ENSANGP27587 (MRP like protein)</td>
<td>1</td>
<td>45</td>
<td>4e-67</td>
<td>C</td>
</tr>
<tr>
<td>7008C03</td>
<td>313</td>
<td>20</td>
<td><em>Canis familiaris</em> (AAS91646)</td>
<td>MRP2</td>
<td>0</td>
<td>36</td>
<td>3e-36</td>
<td>C</td>
</tr>
<tr>
<td>7067D09</td>
<td>397</td>
<td>67</td>
<td><em>Anopheles gambiae</em> (EAA45523)</td>
<td>ENSANGP22549 (RNAseL inhibitor)</td>
<td>2</td>
<td>78</td>
<td>9e-178</td>
<td>E</td>
</tr>
<tr>
<td>7002E01</td>
<td>715</td>
<td>100</td>
<td><em>Xenopus laevis</em> (AAH84777)</td>
<td>LOC398565 protein (ABCF3)</td>
<td>2</td>
<td>49</td>
<td>0</td>
<td>F</td>
</tr>
<tr>
<td>4013B10</td>
<td>344</td>
<td>20</td>
<td><em>Danio rerio</em> (XP_692776)</td>
<td>ABCA3</td>
<td>1</td>
<td>48</td>
<td>9e-82</td>
<td>A</td>
</tr>
<tr>
<td>7052B06</td>
<td>216</td>
<td>29</td>
<td><em>Anopheles gambiae</em> (EAA08957)</td>
<td>ENSANGP19635</td>
<td>1</td>
<td>43</td>
<td>7e-38</td>
<td>H</td>
</tr>
<tr>
<td>mdr7-236</td>
<td>254</td>
<td>21</td>
<td><em>Gallus gallus</em> (XP418636)</td>
<td>Mdr / Pgp-3</td>
<td>1</td>
<td>60</td>
<td>8e-83</td>
<td>B</td>
</tr>
</tbody>
</table>

¹ These sequences have been submitted to GenBank and were assigned the accession numbers DQ146410-DQ146418

² Based on comparison of the length of the contig with its top BLASTp match

³ Number of ATP binding domains determined by searching against the conserved domain database

⁴ As predicted by BLASTp and cluster analysis results
4.3.5 Cluster analysis of ABC transporters from S. scabiei

The EST contigs from S. scabiei all grouped closely with their respective homologues from D. melanogaster and C. elegans (Figure 4.1). The proteins clustered according to predicted ABC-subfamily. Although the designated ABC-H proteins from S. scabiei and D. melanogaster grouped together, the ABC-H from C. elegans instead grouped with ABC-G. The MRP-like proteins from S. scabiei all fell within the ABC-C group. 7001E12 sat apart from other members in this subgroup, supporting the BLASTp result which indicated that 7001E12 was more divergent, whereas 8060B04 and 9002G04 were more similar to MRP 1 and 2 from other organisms. ABC subfamilies B and C were closely related, with group B apparently derived from group C.

4.4 Discussion

In this study we identified nine ABC transporter genes from S. scabiei. ABC subfamilies A, C, E, F and H were represented in the EST database, with an ABC-B protein subsequently identified by further library screening. Cluster analysis found that most S. scabiei contigs clustered closely with their D. melanogaster homologue in each respective subgroup.

Contig 4013B10 was found to belong to the ABC-A subfamily. These are among the largest ABC transporters, averaging 1700aa or more. A. gambiae has six ABC-A proteins, whereas D. melanogaster has 19 (Roth et al., 2003). The physiological roles of these proteins in invertebrates remain unexplored. In humans, ABC-A proteins may be involved in cholesterol transport and have also been implicated in drug resistance (Dean et al., 2001). ABC-A proteins are not present in the yeast chromosome, suggesting they evolved following multicellularity (Sheps et al., 2004). Contig 7067D09 was found to encode the RNAse-L inhibitor protein, belonging to the ABC-E family. This gene is highly conserved and represented as a single copy across most genomes, which suggests an essential housekeeping function. In humans it is involved in the antiviral immune response and is implicated in mRNA turnover. Unlike other ABC transporters, this subfamily contains no transmembrane domains,
Figure 4.1: Dendrogram of *S. scabiei* (Ss) and selected *Drosophila melanogaster* (Dm) and *Caenorhabditis elegans* (Ce) ABC transporter ATP-binding domains. Accession numbers for the sequences used are listed in table 4.3.
and apparently duplicated ATP binding domains. It is consequently often classified as a “non-transport” ABC protein.

The *S. scabiei* 7002E01 sequence displayed homology to the ABC-F protein GCN-20. ABC-F proteins share a similar domain organization to ABC-E, with no transmembrane domains. GCN-20 is involved in translational regulation via the activation of eIF2α (Marton et al., 1997). This group of proteins is well conserved between most genomes studied, with three members each.

An interesting discovery was the assignment of contig 7052B06 to the ABC-H subfamily. This recently discovered group of proteins has been identified in the *D. melanogaster* and *A. gambiae* genomes (Misra et al., 2002; Roth et al., 2003), but is apparently absent from higher eukaryotes. Although there are suggested ABC-H orthologues in *C. elegans*, our results indicate they are phylogenetically distinct from the arthropod protein (Sheps et al., 2004). Nothing is known about the physiological function of this subfamily, but because of its apparent uniqueness to arthropods it has been earmarked as a potential insecticide target (Roth et al., 2003).

Four of the nine *S. scabiei* ABC transporters identified in this study belonged to the ABC-C subfamily. ABC-Cs are well represented across other genomes, with *Homo sapiens* and *Drosophila* containing 12 members, and *Anopheles* having 14 members. ABC-C proteins have been extensively studied due to their implication in multidrug resistance, hence their alternate title of multidrug resistance proteins (MRP). They are closely related to P-glycoproteins with a broad, sometimes overlapping substrate profile. One of the main differences is that MRPs transport substances complexed with glutathione. Additionally, several MRPs are distinct from P-glycoproteins, in that they contain an additional N-terminal transmembrane domain of unknown function. Three *S. scabiei* contigs (8060B04, 9002G04 and 7008C03) were found to have homology to MRPs 1 & 2 from mammals and *Drosophila*. This gene has received recent attention in both *Anopheles* and *Drosophila* genome annotation. Splice variants from a single MRP gene from *Anopheles* can encode ten isoforms (Roth et al., 2003), whereas the *Drosophila* ortholog (CG6214/dMRP) can encode 14 isoforms (Grailles et al., 2003). The implication of the presence of multiple isoforms is not known, but since exon variation occurs in regions thought to be involved in
substrate recognition, these proteins may have an even broader range of substrates, which has implications for drug resistance. Multiple sequence alignments of the S. scabiei ABC-C contigs suggest that they are independent proteins rather than isoforms of a single gene. However, further investigations are needed to fully characterize this important group of proteins in S. scabiei, and particularly to determine whether ivermectin is a substrate.

The identification of a P-glycoprotein (ABC-B) homologue from S. scabiei was highly significant for our search into ivermectin resistance candidates. Our initial survey of the S. scabiei EST database failed to identify P-glycoprotein sequences. However, the EST dataset represents only a small proportion of the library, and P-glycoproteins were subsequently identified from the cDNA library and genomic DNA via degenerate PCR and library screening. The resulting clone had over 50% identity to the C-terminal region of many P-glycoproteins across a range of organisms.

Homologues of several of the ABC transporters identified in this study have been implicated in drug resistance in other organisms and thus are of interest to future studies detailing mechanisms of ivermectin resistance in scabies mites. In humans, the ABC-A3 is found in association with MRP and is also thought to play a role in resistance to anticancer drugs (Klugbauer and Hofmann, 1996).

An important aspect of future research will be to explore, compare and contrast the copy number and expression levels between S. scabiei MRP (ABC-C) and P-glycoprotein (ABC-B), and importantly to determine any significant differences between ivermectin tolerant and susceptible mites. Recent studies have shown evidence of ivermectin exerting selection pressure at a P-glycoprotein gene from Onchocerca volvulus (Ardelli et al., 2005a; Eng and Prichard, 2005). It will be important to determine whether similar selection is observed in our mite populations subjected to multiple doses of ivermectin and presumably under extremely high selection pressure.
Chapter 5

Molecular characterisation of a pH-gated chloride channel from Sarcoptes scabiei

5.1 Introduction

An important target of many insecticides and antiparasitic drugs are the ligand gated ion channels (LGICs). Members of the LGIC family mediate rapid excitatory and inhibitory neurotransmission in muscles and neurons. These channels share a similar hetero-pentameric structure, encoded by subunit proteins consisting of an N-terminal, extracellular ligand binding domain, and four transmembrane domains forming the ion selective pore. A defining feature of all LGICs are paired extracellular cysteine residues, hence they are often appropriately referred to as cys-loop LGICs (reviewed in Bloomquist, 2003).

The superfamily of LGICs can be separated into the excitatory, cation-selective and inhibitory, anion-selective receptors. Neurotransmitters of vertebrate cys-loop LGICs include the cation selective nicotinic acetylcholine and serotonin receptors; and anion selective γ-aminobutyric acid (GABA) and glycine receptors (Ortells and Lunt, 1995). Invertebrates have a diverse range of LGICs, including GABA (ffrench-Constant et al., 1991), serotonin (Ranganathan et al., 2000), acetylcholine (Putrenko et al., 2005), glutamate (Cully et al., 1994) and histamine (Gisselmann et al., 2002) gated chloride channels. In addition, several new clades of chloride channels have recently been identified in insects (Dent et al., 1997; Schnizler et al., 2005), although the neurotransmitters involved are yet to be elucidated.

In nematodes such as Caenorhabditis elegans, the glutamate gated chloride channels (GluCls) appear to be the primary target of ivermectin. In these organisms, binding of ivermectin to GluCls causes irreversible opening of the channels, leading to an influx of chloride ions, hyperpolarisation and paralysis. Ivermectin has also been shown to interact with other invertebrate LGICs, including GABA (Holden-Dye and Walker, 1990), histamine (Georgiev et al., 2002) and pH sensitive chloride channels (Schnizler et al., 2005). The contribution of these subunits to overall ivermectin
toxicity in other nematodes and arthropods remains unclear, and may vary between even closely related organisms.

LGIC alteration has been associated with ivermectin resistance in several studies. Most of these to date have focused on the GluCls, with mutations contributing to ivermectin resistance identified in *Drosophila melanogaster* (Kane *et al.*, 2000), *C. elegans* (Dent *et al.*, 2000) and *Cooperia oncophora* (Njue *et al.*, 2004). Additionally, selection at a GluCl gene in *Haemonchus contortus* was found to be associated with ivermectin resistance (Blackhall *et al.*, 1998b). The potential involvement of GABA-receptors in ivermectin resistance in nematodes has also been reported (Blackhall *et al.*, 2003; Feng *et al.*, 2002).

Having highlighted the importance of LGICs to understanding the physiological mechanism of ivermectin, and therefore the development of resistance, it was critical to identify these genes in the scabies mite. This study reports the isolation and identification of *SsCl*, a novel chloride channel gene from *Sarcoptes scabiei*.

### 5.2 Methods

#### 5.2.1 Isolation of cDNA

The *S. scabiei* var. *hominis* EST database was searched using the *D. melanogaster* glutamate gated chloride channel gene *Glc1* (AF297500) as the query sequence using BLASTn (Basic local alignment search tool) (Altschul *et al.*, 1990). Glycerol stocks from a clone identified as having significant homology (E<0.01) to *Glc1* were obtained and plasmid DNA isolated. Regions of poor sequence quality were re-sequenced using the gene specific primer B08F1 (Table 5.1). Alignment with other LGICs showed the cDNA clone sequence lacked the 5’ and 3’ regions of the gene. To extend the sequence, contig extension PCR was done (Chapter 2) using nested antisense gene specific primers B08R1/R2, followed by B08R3/R4. PCR products were cloned and sequenced.
Table 5.1: \textit{SsCl} sequencing primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
<th>Location in completed sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>B08F1</td>
<td>GCA TCA AAC GTA GTC TAA GC</td>
<td>1019</td>
</tr>
<tr>
<td>B08F2</td>
<td>GAA TTG ATG CCG TAC AAC GA</td>
<td>655</td>
</tr>
<tr>
<td>B08F3</td>
<td>TGA TTT CTA TAT GTC GGG CCA TTT G</td>
<td>597</td>
</tr>
<tr>
<td>B08R1</td>
<td>TTC TGA TAG ACC GAA TAG CC</td>
<td>628</td>
</tr>
<tr>
<td>B08R2</td>
<td>GCA AAT GGC CCG ACA TAT AG</td>
<td>603</td>
</tr>
<tr>
<td>B08R3</td>
<td>CGA TGT CAT GAT AGT AAG CG</td>
<td>201</td>
</tr>
<tr>
<td>B08R4</td>
<td>CGA TCG ATC AAC ATG CTA AC</td>
<td>178</td>
</tr>
<tr>
<td>B08R5</td>
<td>CCA GCT TCA GCA GCT AAT CC</td>
<td>1252</td>
</tr>
<tr>
<td>B08R6</td>
<td>CAG CGA ACC AAA GAT CAA CA</td>
<td>906</td>
</tr>
<tr>
<td>B08R7</td>
<td>TTT CTA TCC AAA AAG AGA TCC ATG A</td>
<td>772</td>
</tr>
<tr>
<td>gGluCIR1</td>
<td>TCG GTC ACC AAT CAA TTT CA</td>
<td>275</td>
</tr>
<tr>
<td>GluCl-F</td>
<td>ATG TTT TTG AAG CAA AAA TTA TAT C</td>
<td>1</td>
</tr>
<tr>
<td>GluCl-R</td>
<td>TTA CAA ATA TGA CCA ATG AAT TAG</td>
<td>1447</td>
</tr>
<tr>
<td>ClNdeI</td>
<td>TAT CAT ATG ATG TTT TTG AAG CAA</td>
<td>1</td>
</tr>
<tr>
<td>ClXbaI</td>
<td>CAT CTA GAT TAC AAA TAT GAC CA</td>
<td>1447</td>
</tr>
</tbody>
</table>

Sequence alignments indicated that the extended cDNA contig was still incomplete at the 5’ and 3’ ends. Rapid Amplification of cDNA Ends (RACE) was subsequently employed to obtain the complete sequence. First-strand synthesis for preparation of 5’ and 3’ RACE ready cDNA was done as described in chapter 2.

RACE PCR was performed using the BD Advantage 2 PCR kit. Reactions contained 1 X PCR buffer, 0.2mM dNTPs, 1X polymerase mix, 1 X universal primer mix (UPM), 0.2µM gene specific primer (GSP), 2.5 µL RACE-ready cDNA and dH$_2$O to a final volume of 50 µL. For 3’ RACE, three different GSPs were used- B08F1,
B08F2 and B08F3 (Table 5.1). A positive control used the GSPs B08F3/B08R6, and a negative control contained UPM only. Reactions were cycled 40 times at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 3 minutes. PCR products were diluted 1:10 in Tricine-EDTA buffer and 5µL used for a second round of PCR with a nested universal primer (NUP) and nested GSPs. 5’ RACE reactions were performed as described above, with the first round employing the gene-specific primers B08R1, B08R3 and B08R6. First round PCR products were diluted 1:100 in Tricine-EDTA, and nested PCR performed. PCR conditions were as above except annealing temperature was increased to 62°C and cycle number reduced to 35. Second round positive products were cloned and sequenced.

To amplify the full length sequence, the primers GluCl-F and GluCl-R (Table 5.1) were designed from the 5’ and 3’ RACE clone sequences, and a semi-nested PCR performed using the 5’ RACE ready cDNA as template. Reaction components were as described previously. The first round used the primer combination UPM and GluCl-R. Reactions were cycled 40 times at 94°C for 30sec, 55°C for 30sec and 72°C for 2min. PCR products were diluted 1:50 in Tricine-EDTA, and 5 µL used in the second round with the primers GluCl-F/GluCl-R. Reactions were cycled as above except annealing temperature was reduced to 50°C. Second round PCR products of the anticipated size were cloned and sequenced.

5.2.2 Isolation of genomic DNA
A PCR-based library screening technique using the primers B08F2/R5 was used to identify a clone from the *S. scabiei* genomic DNA library (chapter 2). Following three rounds of PCR screening and phage enrichment, the PCR positive well was plated as plaques and PCR performed on individual plaques. Phagemids were excised from positive plaques, purified and sequenced with the following primers: T3, B08F1, B08F2, B08R1, B08R3, B08R5, B08R6, gGluClR1.

5.2.3 Screening the cDNA library for additional LGIC subunits
To identify additional LGIC-like subunits in the cDNA library, plaque hybridizations were performed. Plating of Yv7 libraries and filter lifts were described previously (chapter 2). Ten filters (approximately 5 X 10^5 pfu) were screened in the primary round. The primers B08F2 and B08R5 were used to generate a 650bp product
CHAPTER 5

spanning the M1-3 region which is well conserved across LGIC family members. The PCR product was purified and labeled α-32P dCTP. Hybridisations, washes and X-ray exposure are described in chapter 2, and were performed under relatively low stringency conditions (50°C).

5.2.4 Sequence analysis

BLASTp and conserved domain searches were conducted on the SsCl amino acid sequence via the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Transmembrane domains were predicted using TMPred (Hofmann and Stoffel, 1993) (accessed via http://www.ch.embnet.org/software/TMPRED_form.html), and SignalP v3.0 was used to identify potential signal sequences (Nielsen and Krogh, 2004). N-glycosylation and protein kinase phosphorylation sites were predicted via programs available via the CBS prediction servers (http://www.cbs.dtu.dk/services/).

To elucidate the relationship of SsCl to other chloride channels, multiple sequence alignments between SsCl and Drosophila chloride channels was performed. D. melanogaster anion-selective ligand gated ion channels were identified using Flybase (www.flybase.bio.indiana.edu) and by referral to the recent analysis of Drosophila LGICs by Prof. Joseph Dent (Dent, 2006). Amino acid sequences were aligned using ClustalW and bootstrapping for confidence determination performed using Seqboot. Neighbour-joining trees were constructed using Protdist and Neighbour (Felsenstein, 1989).

5.2.5 Homomeric expression of SsCl in Xenopus oocytes

Cloning sites were incorporated into the full-length SsCl open reading frame by PCR with the primers ClNdeI and ClXbaI, (flanking the 5’ & 3’ ends respectively) (Table 5.1). This product was subcloned into pGEM-T Easy before cloning into the pT7TS expression vector. After confirmation of correct sequence and orientation, the plasmid was sent to Prof. Dent’s laboratory at McGill University.

The pT7SsCl construct was linearised with BamHI and capped cRNA generated using the MEGAscript transcription kit (Ambion, Austin, TX, USA). SsCl cRNA was precipitated with LiCl and resuspended to a final concentration of 1µg/mL in RNAse free H2O.
**Xenopus laevis** oocytes were harvested using standard methods (Goldin, 1992) and maintained under the conditions described by (Putrenko et al., 2005). *SsCl* cRNA (approx 40nL) was micro-injected into oocytes using the Nanoject system (Drummond Scientific). cRNA was injected with and without a cRNA encoding a GFP marker as a control to ensure oocytes were expressing correctly.

Oocytes were analysed by two-electrode voltage clamp with a fast perfusion system using a Maltese Cross chamber (ALA Scientific Instruments, Westbury NY). Recordings were sampled at 1kHz using Clampex 8.1 digital oscilloscope software (Axon Instruments, Foster City CA.) as described by Putrenko (2005). Neurotransmitters tested (GABA, glutamate, glycine, acetylcholine, serotonin, octopamine, tyramine, histamine, dopamine and zinc, 1mM, pH 7.5) were obtained from Sigma-Aldrich (Oakville, ON).

pH response curves were fitted using the Hill equation: \( f(I) = (I_{\text{max}}[I]^n/(EC_{50}[I]^n) + I_{\min}) \), where \( I = \) response normalized to the maximum response (pH 9), and \( I_{\text{max}} = \) estimated maximum response. \( EC_{50} = \) pH eliciting half-maximal response, and \( I_{\min} = \) estimated minimum response, were free parameters. pH changes were effected by fast perfusion of oocyte with solutions of pre-measured pH. To determine whether the channel was anion selective, current-voltage (I-V) curves were generated using voltage ramps in ND96 (96mM NaCl, 2mM KCl, 1.8mM CaCl₂, 1mM MgCl₂, 5mM Hepes) or in ND96 with sodium gluconate substituted for NaCl (7.6 mM chloride). For each oocyte, a voltage ramp (4 mV/s) was performed at pH 6.0 (channels closed) and subtracted from a ramp at pH 7.0. The bath electrode was embedded in an agar bridge containing 3M KCl to avoid effects of changing chloride concentration on its electrochemical potential. The pH of the ND96 was changed by adding HCl or NaOH.

### 5.3 Results

#### 5.3.1 Isolation of *SsCl* cDNA

The cDNA library clone Yv7069B08 showed significant homology to members of the LGIC family. The Yv7069B08 EST sequence of 826bp was extended to 921bp after resequencing of the plasmid. A PCR approach combining vector primers with
the nested reverse primers B08R1/R2, R3 and R4 successfully extended the 5’ end of
the cDNA contig by 404bp. This 1325bp contig was called ‘B08R4’ (Figure 5.1).

Following two rounds of 3’ RACE PCR, two fragments were cloned and sequenced
(Figure 5.2). The first was 0.6kb, derived from the first round PCR with primers
UPM/B08F2 and nested with NUP/B08F1. The second fragment was 0.95kb,
obtained from the first round combination of UPM/B08F3 followed by NUP/B08F2.
This aligned contig (named RCl.F) was 937bp, which added an extra 258bp to the 3’
end of the B08R4 contig (Figure 5.1). A putative stop codon was identified, followed
by a 3’ UTR of 96bp and a polyA tail. The first round of 5’ RACE PCR amplified
several bands of interest with the UPM/B08R3 combination. Following a second
round with NUP/B08R4, a 283bp fragment was cloned and sequenced (Figure 5.2).
This sequence (named RCl.R) aligned with the B08R4 contig, adding an extra 163bp
to the 5’ end of the cDNA (Figure 5.1). An 86bp 5’ UTR preceded the putative start
codon. After identification of the 5’ and 3’ ends using RACE, the full length cDNA
was amplified from the 5’ RACE ready cDNA using two rounds of PCR. The
resulting clone, designated SsCl, was sequenced, resulting in a full length cDNA
sequence of 1470bp (Figure 5.2).

5.3.2 Identification of the SsCl genomic DNA sequence

Three rounds of PCR-based screening and amplification enriched the S. scabiei
genomic DNA library for SsCl phage. The third round positive well was plated as
plaques, of which 1/10 were positive by PCR. This phage (designated pNYF2R5)
was excised from the library and estimated by BamHI digestion to be about 5kb.
1742bp of this aligned with the B08R4 and RACE cDNA sequences (Figure 5.1).
Four introns were identified in the genomic sequence (Figure 5.3). Alignment of the
genomic DNA with the two cDNA contigs revealed that there were several errors in
the B08R4 sequence, with a premature stop codon and what appeared to be
incorrectly spliced exons (Figure 5.3).
Figure 5.1: Sequencing strategy for obtaining the full length SsCl gene.
The contig was completed through a combination of cDNA library clones, contig extension PCR, genomic library screening, and RACE. (schematic representation only, not to scale).
Figure 5.2: 5’ & 3’ RACE amplification products.
(a) 3’ RACE nested PCR. First round products from universal (UPM) and gene specific primers (B08F1, F2 & F3) were diluted and amplified with nested universal (UPM) and gene specific primers. (*) denotes products cloned and sequenced.
(b) 5’ RACE nested PCR.
(c) Amplification of the full length SsCl cDNA. Lane 1: First round PCR with Universal primer mix and GluCl-R. Lane 2: 1/50 dilution of first round PCR product, amplified with GluCl-F/GluCl-R primers.
gDNA clone
B08R4
SsCl

> intron 1

> intron 2
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gDNA clone           AACAATTGTTGATAGAGATATTTCTCTATATGTCTGGGCCATTTTGGCTAGAAAGG
B08R4               AACAATTGATAAGAGATATTTCTCTATATGTCTGGGCCATTTTGGCTAGAAAGG
SsCl                AACAATTGATAAGAGATATTTCTCTATATGTCTGGGCCATTTTGGCTAGAAAGG

gDNA clone           CTATTCGGGTCTATCCAGAAAGATGTTGCTATTTGCTGAAAGG
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SsCl                CTATTCGGGTCTATCCAGAAAGATGTTGCTATTTGCTGAAAGG

gDNA clone           ATAGCGCCTTATTTGTTCATCTTCATCTTAAGCGGAAATTTTATCAT
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gDNA clone           ATCCTGTTTTGGATGAAATCACTTGTATACCAGCAAGAGTAACACTTTGTG
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gDNA clone           TGACAACATTGCTAGCAATGGTGACTGTTTCGAAGGAATCCAAACAAAAT
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gDNA clone           ATTCCAAAAGTGCCATATGTCAAAGCTGTTGATCTTTGGTTCGCTGGTTG
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> intron 4

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SsCl                TTGAATCAATAGCAAGATCGTATCGCTCCCAGACGTTTCAGCAGTAACTG

99
Figure 5.3: ClustalW alignment of *SsCl* genomic and cDNA sequences.
The positions of introns 1-4 in genomic DNA are indicated (>). Nucleotides differing from the consensus are shaded grey. Altered regions of the cDNA library contig B08R4 are underlined. Start (ATG) and stop (TAA) codons are shaded blue.
5.3.3 cDNA library screening for additional LGIC subunits

After two rounds of screening, five hybridisation positive clones were excised and sequenced. These were selected on the basis of varying hybridisation signals, because the degree of similarity between the probe and potential LGIC homologue was unknown. Selecting all strong signals may only result in clones identical to the probe, while selecting all weak signals may not identify anything significant. Unfortunately, no new relevant sequence information was obtained from these results. From the five clones excised, only one showed homology to LGICs, and this was identical to the original 7069B08 sequence (data not shown).

5.3.4 SsCl sequence analysis

The SsCl nucleotide sequence was predicted to encode a protein of 489aa (Figure 5.4). A neurotransmitter-gated ion-channel transmembrane region domain was identified over amino acid residues 247-486, and the ligand binding domain was identified over residues 32-240. Two cysteines separated by 13aa, characteristic of cys-loop LGICs, were present along with conserved N-glycosylation and phosphorylation sites. A signal sequence was predicted over residues 1-25. The sequence did not however contain the second pair of cysteine residues commonly observed in glutamate, histamine and glycine channels (double cys-loop LGICs) (Dent, 2006). Four transmembrane domains were predicted over the C-terminal half of the protein, with a long, poorly conserved region between TM 3-4 as seen with other LGICs. A pro-ala-arg motif was observed in TM2, which was indicative of an anion selective channel (Galzi *et al.*, 1992).

BLASTp results showed the sequence had 25-30% identity to many members of the ligand gated ion channel family, particularly the GABA, glycine, and glutamate receptors. However at such low identity levels it was not possible to assign the SsCl sequence to any particular sub-family upon Blast results alone. Phylogenetic analysis using the neighbour-joining method placed the SsCl sequence between the recently identified *Drosophila* group 1 and pH sensitive chloride channel groups. The GABA, histamine, and glutamate receptors formed distinct clades as expected (Figure 5.5).
Figure 5.4: Sequence alignment of SsCl with *D. melanogaster* pH sensitive and glutamate gated chloride channels.

SsCl was aligned with pHCl-A (AAX11175) and Glc1 (AAG40735). Black shading indicates identical residues, and grey shading similar residues. Transmembrane segments 1-4 (______) and putative signal peptide (----) are underlined. Conserved cysteine residues (♦), predicted N-glycosylation (•) and protein kinase phosphorylation (°) sites are indicated. The boxed PAR motif in the TM2 pore forming region predicts anion selectivity.
Figure 5.5: Neighbour joining tree showing relationship of SsCl to *Drosophila melanogaster* chloride channel subunits.

SsCl was aligned with the following *Drosophila* ligand gated chloride channels: GABA (AAB27090, NM132862, CAA55144, AAA28556); Histamine (NP_731632, AAF55691, AAF58743); Group 1 (AAF57144, AAF49337, AAF45992); pH sensitive (AAX11175); Glutamate (AAG40735). Numbers to the left of each branch indicate bootstrap levels from 100 replicates. SsCl clusters with the recently discovered insect Group 1 and pH sensitive clades, of which the ligand neurotransmitter is unknown.
5.3.5 Functional characterisation of SsCl

SsCl formed a homomeric channel whose current was dependant on the extracellular pH (Figure 5.6). The channel is closed at pH 6.0 and maximally activated at pH 9.0 (Figure 5.6a). This pH dependence was demonstrated in a dose-dependant manner by fit to Hill equation, with a half-effector pH (EC$_{50}$) of 7.55 ± 0.06 (Figure 5.6b). pH dependant currents were not observed in control oocytes. No response was seen in oocytes to the neurotransmitters GABA, glutamate, glycine, acetylcholine, serotonin, octopamine, tyramine, histamine, dopamine or zinc.

To confirm the anion selectivity predicted from the amino acid sequence, we generated $I-V$ curves in the presence and absence of chloride (Figure 5.6c). The reversal potential in the presence of chloride was -31.4 ± 3.4 mV, whereas the reversal potential in the absence of chloride was 24.3 ± 4.1 mV. The difference of 55.7 ± 5.3 mV is consistent with the shift for chloride channels of 58mV predicted by the Nernst equation.

The response of SsCl to ivermectin was also tested. Ivermectin activated the SsCl channels, even at pH 5.5 when the channels should be predominantly closed (Figure 5.7a). The response to ivermectin increased gradually and reached a maximum current greater than the maximum response to pH 9.0 in the absence of ivermectin (data not shown). The current did not return to baseline despite washing with drug free media, indicating that activation of the channel by ivermectin was irreversible. Increasing pH appeared to potentiate the ivermectin response, as the response to ivermectin reached its maximum more rapidly at pH 7.0 than at pH 5.5 (Figure 5.7b).
Figure 5.6: SsCl forms a homomeric pH-gated chloride channel when expressed in *Xenopus* oocytes.

a) Representative traces from voltage-clamped oocytes expressing SsCl showing the current response to increases in pH. The baseline pH is 6.0. Oocytes were clamped at -80 mV.

b) Curve showing the increase in current response with increased pH. The current was normalised to the current at pH 9.0 for each oocyte. The curve represents a fit to the Hill equation. n=4, error bars represent standard error of the mean. Oocytes were clamped at -80 mV.

c) Current-voltage relationship in “high” (103.6mM) and ”low” (7.6mM) external chloride. Both curves for each oocyte were normalised to the current in high chloride at a membrane potential of -70mV. n = 4, error bars represent standard error of the mean.
Figure 5.7: SsCl is activated by ivermectin.
a) Trace of oocyte in pH 5.5 medium perfused with 10µg/mL ivermectin in pH 5.5 medium. Ivermectin was washed out with ivermectin free pH 5.5 medium.
b) In this trace oocyte started in pH 6.0 medium, was perfused with pH 7.0 medium and then pH 7.0 medium plus 10µg/mL ivermectin. The ivermectin was washed out with pH 7.0, then pH 6.0 medium. Note the different time scale from the trace above. In both t, oocytes were clamped at -80 mV.

5.4 Discussion

We report the molecular characterisation of a novel chloride channel gene from *S. scabiei*. SsCl shows sequence and structural characteristics of an invertebrate cyst-loop ligand gated chloride channel, however BLASTp and phylogenetic analysis did not suggest strong homology to the well characterised glutamate, histamine or GABA gated chloride channels. Instead, SsCl clusters between the *Drosophila* pH-sensitive (pHCl) and group 1 clades. The group 1 clade was recently identified through bioinformatic analysis of the completed *Drosophila* genome (Dent, 2006). pHCl and Dm group 1 orthologs have also been reported in other insects such as *Anopheles* and *Apis mellifera* (Jones and Sattelle, 2006). This study is the first confirmation that this group exists outside the diptera.
We investigated the pharmacological properties of SsCl by expression in *Xenopus* oocytes. No current response was elicited to any of the neurotransmitters tested. This was not surprising given its phylogenetic grouping. pHCl was recently characterised in *Xenopus laevis* oocytes and found to have pharmacological properties unique to LGICs. The channel was not responsive to any of the neurotransmitters tested, however was extremely sensitive to extracellular pH and importantly, was activated by ivermectin (Schnizler et al., 2005). It is not known whether any group 1 homologues have been tested for similar pH dependence.

Our results show clearly that SsCl is also a pH-gated chloride channel. The channel was sensitive to changes in extracellular pH, and displayed a similar pharmacological profile to its *Drosophila* ortholog. Significantly, SsCl channels expressed in *Xenopus* oocytes were activated by ivermectin, even at acidic pH when the channel is usually closed. However in contrast to the *Drosophila* pHCl, currents did not return to baseline after ivermectin was washed out. This irreversible activation is consistent with that observed for glutamate-gated chloride channels. However, the concentrations of ivermectin required to activate glutamate gated chloride channels in other organisms are up to 10,000-fold lower than observed for SsCl (Cully et al., 1994). Due to time constraints, the minimal and and maximal concentrations of ivermectin required to activate SsCl were not determined. This is an important consideration to be evaluated in future studies, particularly in context of the levels of ivermectin exposure *in vivo*.

Unusually, protons appear to inhibit these channels even in the absence of an endogenous ligand. It was suggested that ivermectin was responsible for gating pHCl in *D. melanogaster*, with pH modulating the response of the channel to ivermectin (Schnizler et al., 2005). Our results support this contention, but since ivermectin is not native to the organism, it is quite possible that a currently unidentified neurotransmitter binds to pHCl. It has also been proposed that these pH modulated subunits may exist in vivo within a heteromultimeric channel (Schnizler et al., 2005). Co-expression with one or more different LGIC subunits may explain why they are not activated by any of the conventional ligands in their homomeric state.

In this study several approaches were taken to identify the full length cDNA of SsCl. We initially used an EST database derived from a *S. scabiei* cDNA library to obtain
the partial sequence. Because the library was constructed with oligo-dT, truncation at 5’ ends is commonly observed, and thus further work was needed to complete the sequence. The 3’ end of the B08R4 contig was initially thought to be complete due to the presence of an apparent poly-A tail. However comparison to other sequences and the absence of the M4 region revealed that the polyA region was actually a result of oligo-dT mispriming. There were additional anomalies with the B08R4 sequence, with apparent altered exon splicing when compared with the RACE and genomic DNA sequences. For example in B08R4, intron 3 remained unspliced, intron 4 had different splice sites, and part of exon 5 was missing. Alternate splicing, particularly in this region of the protein is commonly observed in other chloride channels (Jones and Sattelle, 2006; Schnizler et al., 2005; Semenov and Pak, 1999), and is thought to contribute to subunit diversity and channel kinetics (Hosie et al., 1997). However these alterations to B08R4 result in a truncated protein, so whether these represent true splice variants or simply artefacts of cDNA library construction and PCR based normalisation (Fischer et al., 2003b) is uncertain. It is possible that this sequence actually corresponded to pre-mRNA rather than spliced mRNA, which has been observed in several other *S. scabiei* EST library clones. Although RT-PCR experiments to date have not noted any obvious size variations in transcripts, more targeted RT-PCRs using different primer combinations may help address this question.

By analogy with other arthropods, it is highly likely that SsCl represents just one of many LGICs in *S. scabiei*. Unfortunately there is virtually no sequence data on arachnid chloride channels available in public databases, making gene identification difficult. We attempted to identify other chloride channel groups via hybridisation based cDNA library screening, which has been used to successfully identify LGICs previously (Zheng et al., 2003), however we were unsuccessful, despite a number of positive plaques observed during primary screening. The limited success of secondary screening can probably be attributed to diffusion of phage in the interval between filter lifts and phage isolation, which occurred several days apart (data not shown). Conversely, the relatively straightforward PCR based library screening was successfully used to identify the corresponding genomic DNA sequence of SrCl. The limitation of this approach is that at least a small region of sequence must be known in order to design PCR primers. Interestingly, previous attempts to isolate GluCl
transcripts in *Tetranychus urticae* mites using *Drosophila* GluCl probes under low stringency conditions were unsuccessful, suggesting that mite GluCls may be quite divergent (Cully *et al*., 1996). In order to identify further LGICs from *S. scabiei*, degenerate PCR utilising conserved M2 regions may be useful, particularly as more sequence information from other species becomes available.

Our results provide evidence that SsCl may be a target for ivermectin activity in the scabies mite. It may therefore be of considerable relevance to the emergence of ivermectin resistance. Since nothing is currently known about the physiological role of these novel channels in scabies mites or any arthropod, further characterisation is critical. Of particular relevance would be immunohistochemistry studies to determine the localisation patterns of this channel, its possible co-expression with other subunits, and its function in the arthropod nervous system.

In addition, quantitative RT-PCR analysis (chapter 6) will provide information on the expression of *SsCl* in different life stages and ivermectin exposure levels in *S. scabiei*, which is important when considering any possible interaction with ivermectin. Previous studies indicate that ivermectin may exert selection pressure on nematode chloride channels (Blackhall *et al*., 1998b; Blackhall *et al*., 2003; Njue and Prichard, 2004), therefore single-strand conformational polymorphism (SSCP) analysis will be undertaken (chapter 7) to see if similar selection is observed in *S. scabiei*.

In summary, SsCl represents a novel class of ligand gated chloride channel in arthropods. The identification of this gene contributes significantly to investigating ivermectin activity in scabies. Future work will further explore the interaction of ivermectin with SsCl, thus evaluating its contribution to potential drug resistance.
Chapter 6

Relative transcription of *Sarcoptes scabiei* candidate ivermectin resistance genes

6.1 Introduction

The development of drug resistance is commonly associated with alterations in gene expression. For example, metabolic detoxification and increased drug efflux occur via increased enzymatic and transporter activity, which would be evident at the transcriptional level. Although target site alteration may be mediated by mutation induced conformational changes; it may also manifest as a change in drug binding site availability due to an overall increase or decrease in protein. Therefore, analysis of gene expression is of considerable importance to understanding the molecular events underlying the emergence of drug resistance.

The transcription of ABC transporters has been investigated in several parasites and their model organisms. In *Caenorhabditis elegans* for example, semi-quantitative RT-PCR showed that MRP transcription was stable throughout development, with a slight peak in early larval stages (Broeks *et al.*, 1996). Similarly, in *Drosophila melanogaster*, MRP transcription was highest in young embryos (Tarnay *et al.*, 2004). Recent studies on the protozoan parasite *Leishmania* used custom microarrays and qRT-PCR to profile the transcription of ABC transporters in different developmental stages, and demonstrated that three different classes of ABC transporters were up-regulated in antimonial resistant strains (Leprohon *et al.*, 2006). In regard to ivermectin resistance however, these approaches have not been extensively applied, despite some very promising early findings. In *Haemonchus contortus*, northern blotting experiments demonstrated increased P-glycoprotein transcription in ivermectin resistant strains (Xu *et al.*, 1998). Semi-quantitative RT-PCR on *Onchocerca volvulus* P-gp homologues found elevated transcription in adult worms, which was proposed to explain differential ivermectin sensitivity between life-stages (Huang and Prichard, 1999). However, in both cases, these early observations have not been validated with more detailed expression and/or functional studies.
Information on *in vivo* expression levels of ligand gated chloride channels in parasites is also scant. Localisation and drug binding studies suggest these transcripts are developmentally regulated, which in turn has implications for drug activity and resistance. For example, one report showed large differences in glutamate binding between larval and adult stages of *H. contortus*, which suggested that GluCl transcription may be up regulated in larvae (Paiement *et al.*, 1999), but again molecular studies are lacking.

There has been considerably more work done on the association of glutathione S-transferase (GST) expression with drug resistance. The roles of GSTs include metabolism of toxic compounds, digestive processes and modulation of intracellular transport (Wilce and Parker, 1994). A “detox chip” microarray was developed to survey for insecticide resistance in *Anopheles gambiae*, showing up-regulation of GSTs (David *et al.*, 2005). Elevated levels of GSTs have been found in DDT and pyrethroid resistant *Aedes aegypti* (Lumjuan *et al.*, 2005). GSTs also comprise an important group of allergens in many organisms, including house dust-mites (*Dermatophagoides pteronyssinus*) (O’Neill *et al.*, 1995) and intestinal helminths (Spithill *et al.*, 1997). Several GSTs have been identified from *Sarcopes scabiei* var. *hominis*, and were found to correspond to mu and delta classes. One protein of the mu class was characterised further and found to be a major allergen in crusted scabies (Dougall *et al.*, 2005). Additionally, a delta class GST has been characterised from *S. scabiei* var. *vulpes* and found to localise to the mite integument (Pettersson *et al.*, 2005). However, there are no reports regarding in vivo expression levels of scabies mite GSTs or their possible contribution to drug resistance.

Quantitative reverse transcriptase PCR (qRT-PCR) is a powerful and increasingly popular tool for evaluating gene transcription. It is different to conventional PCR in that amplification and detection occur in a single step, meaning data is collected during PCR, rather than the end-point of the reaction. This is enabled by use of fluorescent chemistries that relate fluorescence intensity to PCR product concentration. qRT-PCR has several benefits over more traditional methods of mRNA quantification such as RNase protection assays or northern blotting. It has a wider dynamic range, and is much more sensitive, capable of detecting even a single transcript (Palmer *et al.*, 2003). Changes in transcription levels can be detected more
accurately than techniques such as semi-quantitative RT-PCR and band densitometry (Schmittgen et al., 2000). Most importantly, the technique requires much less RNA than traditional methods.

Apart from estimates of relative EST abundance, gene transcription studies have not been undertaken in *S. scabiei*, due to limited availability of genetic material. However, the above advances mean that we are now in a position to capitalise on recent progress in scabies gene discovery with more detailed molecular studies. In this chapter, qRT-PCR was undertaken to further characterise genes of interest to ivermectin resistance in *S. scabiei*. These included representative ABC transporter, chloride channel, and glutathione S-transferase genes. The first objective was to determine the levels at which candidate genes were expressed in the scabies mite, and whether transcription was developmentally regulated. Secondly, transcription was compared between untreated and ivermectin exposed mites, to determine whether gene transcription was correlated with drug exposure.

### 6.2 Methods

#### 6.2.1 Source of mites

*S. scabiei* var. *hominis* mites were collected from the skin of crusted scabies patients (chapter 2), and drug treatment status at the time of mite collection recorded. Live mites were stored in pools of approximately ten, separated according to life stage (larvae, nymph, adult male, adult female) wherever possible. In total, 553 mites were collected from six different crusted scabies patients during 2005 and 2006. Of these 243 were collected from patients prior to treatment, and 310 mites had been exposed to one or more doses of ivermectin (Table 6.1).

#### 6.2.2 RNA extraction and reverse transcription

RNA was extracted and DNase I treated to reduce contaminating genomic DNA (chapter 2). Attempts to quantify RNA using by spectrophotometry (chapter 2) were unsuccessful; most samples were below the range of accuracy of the instrument, so quantification was not routinely performed. RNA was reverse transcribed to cDNA using the sensiscript RT kit (Qiagen), which is optimised for total RNA yields of
Table 6.1: *S. scabiei* RNA samples used in this study

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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Samples were coded according to patient, number of mites in sample and life stage

^b Patient status at time of collection. 0= no treatment, 1= 1 dose of ivermectin, 2= 2 doses of ivermectin, 3= 3 or more doses of ivermectin (nb. Third dose of ivermectin was given with topical benzyl benzoate)
50ng or less (chapter 2). RT reactions were diluted 1:5 in dH2O prior to use in PCR.

### 6.2.3 qRT-PCR design and optimisation

#### 6.2.3.1 Primer design

Eight genes of interest were investigated (Table 6.2). These included the multidrug resistance proteins and P-glycoprotein described in chapter 4, and the chloride channel described in chapter 5. Additionally, two *S. scabiei* glutathione S-transferases previously described (in Dougall et al., 2005) were examined. β-actin was included as a reference gene for calibration and normalisation purposes. PCR primers were either chosen from existing primers or designed with the assistance of Primer3 software (Rozen and Skaletsky, 2000) ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). Since all reactions were to be carried out using the same cycling conditions, it was important that amplification efficiency was similar for all different genes. Thus primers were selected on the basis of similar Tm values and fragment length (200-300bp). Primer sequences were queried with BLASTn to check that non-specific binding of human cDNA or co-amplification of multiple *S. scabiei* ABC transporters did not occur. To determine optimal cycling conditions, primers were tested on the Yv7 cDNA library. For several primer sets, optimisation was carried out on the Smart Cycler real time PCR machine (Cepheid, Sunnyvale, CA, USA), which has a gradient function. The annealing temperature that gave the lowest cycle threshold (Ct) and the maximum specificity for the most genes was selected.

#### 6.2.3.2 Determination of PCR amplification efficiency

For accurate quantification of mRNA, it is important to determine the PCR efficiency for each amplicon. Because many PCRs do not have ideal or equal amplification efficiency, not correcting for it may lead to incorrect estimations of starting concentration. This is particularly important in the case of low abundance transcripts (Cts >26), where small changes in efficiency may result in large differences in PCR product concentration (Freeman et al., 1999). To determine efficiency, real time PCR was performed on linearised plasmid cDNA clones for each of the nine genes investigated (Table 6.3). Plasmids were quantified and serially diluted 1:10 in dH2O. At least five dilutions were used to construct the standard curve. PCR was done using the QuantiTect SYBR green PCR kit (Qiagen). Reactions contained 1 X SYBR green
master mix, 0.4µM primers, 1 µL template and dH₂O to total volume of 10 µL. Reactions were cycled in the Corbett Rotor Gene 2000 real-time cycler (Corbett Research, Australia). Cycling conditions were: initial denaturation 95°C for 15 min, followed by 45 cycles of 94°C, 15 sec; 56°C, 30 sec; 72°C, 30 sec; with data acquisition at 76°C, 20 sec. Standard curves and efficiency calculations were produced using the Rotor Gene software, which uses the equation Efficiency= -1/S, where S is the slope of the line produced by Ct of the serial dilutions.

Table 6.2: Primer sequences for qRT-PCR studies

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Fragment size (bp)</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1</td>
<td>257</td>
<td>B04F2</td>
<td>CGG TGT CAA ACT TTC CGT CT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B04R3</td>
<td>ATC GCT AAA GCA CCG ATC AC</td>
</tr>
<tr>
<td>MRP2</td>
<td>231</td>
<td>G04F2</td>
<td>GTT GGC TTC AAG TTC GCC TA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G04R3</td>
<td>GCT TCC GGA ACA ACA TCA GT</td>
</tr>
<tr>
<td>MRP3</td>
<td>219</td>
<td>C03F3</td>
<td>CAC CCA ATC CCA TAA GAA TGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C03R3</td>
<td>TGA TGA CCG TTT TCG TAG GG</td>
</tr>
<tr>
<td>MRP4</td>
<td>218</td>
<td>MRP4b-F</td>
<td>TCT CAT CCG AAG ACA TCC AA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MRP4b-R</td>
<td>CTC CCA TCT CTC CAT CAA GC</td>
</tr>
<tr>
<td>P-gp</td>
<td>155</td>
<td>236F2</td>
<td>AGG CAA CTT CAG CAC TCG AT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>236R5</td>
<td>ACA TTC TGA CCG CCA TCA AT</td>
</tr>
<tr>
<td>SsCl</td>
<td>329</td>
<td>B08F3</td>
<td>TGA TTT CTA TAT GTC GGG CCA TTT G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B08R6</td>
<td>CAG CGA ACC AAA GAT CAA CA</td>
</tr>
<tr>
<td>GST-1 (mu)</td>
<td>228</td>
<td>A08F1</td>
<td>GCT ATT GGG ATC TTC GTG GA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A08R1</td>
<td>TGC CCA AAT ACC GGA GAA TA</td>
</tr>
<tr>
<td>GST-2 (delta)</td>
<td>244</td>
<td>A06F1</td>
<td>ATG GAG GTG GTT TGA ACG AG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A06R1</td>
<td>TCG TGA TCG ACA GCA TTC AT</td>
</tr>
<tr>
<td>β-actin</td>
<td>311</td>
<td>5805-F</td>
<td>CAA CCA TCC TTC TGT GGT ATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5805-R</td>
<td>CCA GCT TCG TCG TAT TCT TGT</td>
</tr>
</tbody>
</table>
Table 6.3: cDNA plasmid clones used for determination of qRT-PCR efficiency

<table>
<thead>
<tr>
<th>Gene</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1</td>
<td>Yv8060B04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRP2</td>
<td>Yv9002G04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRP3</td>
<td>Yv7008C03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRP4</td>
<td>Yv7001E12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pgp</td>
<td>pMDR236-3A2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SsCl</td>
<td>pT7SsCl&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GST1</td>
<td>pGST1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>GST2</td>
<td>Yv4002A06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B-actin</td>
<td>Yv6010H03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Obtained from *S. scabiei* cDNA clone collection;  <sup>b</sup> Chapter 4;  <sup>c</sup> Chapter 5;  <sup>d</sup> From (Dougall et al., 2005)

6.2.3.3 Confirming identity of qRT-PCR products

To confirm primer specificity and identity of amplified cDNAs, products from each primer combination were sequenced. At the completion of qPCR, selected samples were diluted 1:100 in dH<sub>2</sub>O and 1 μL used as a template in a second round of conventional PCR. PCR products were sequenced with the gene-specific forward primer (Table 6.2). Sequences were aligned with the existing *S. scabiei* cDNA sequences using the BLAST2 sequences program (Tatusova and Madden, 1999) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

6.2.4 Real time PCR on scabies mite cDNA

For each scabies mite cDNA template, real time PCR was performed for the eight target genes of interest. Each run included amplifying the gene target in parallel with β-actin. This allowed for normalisation of varying cDNA concentrations and gave an indication of inter-assay reproducibility. Each PCR included Yv7 cDNA as a positive control and dH<sub>2</sub>O as a no template control. Reactions were performed in duplicate for β-actin, and quadruplicate for the target gene (Figure 6.1). Individual reactions contained 1 X SYBR green master mix, 0.4μM primer mix, 3 μL diluted cDNA template and dH<sub>2</sub>O to final volume of 10 μL.
**Figure 6.1: Overview of real-time PCR reaction set up.**

For each cDNA sample, two β-actin (reference gene), and four target gene replicates were performed per PCR run. A no template control (NTC) reaction contained dH₂O only, and a positive control contained Yv7 cDNA. No RT controls using RNA template and β-actin primers assessed samples for genomic DNA contamination.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA: WB10L1</td>
<td>WB10L2</td>
<td>WB10N1</td>
<td>WC10N2</td>
<td>WB10Ma1</td>
<td>WB10Ma2</td>
<td>WB10F1</td>
<td>WB10F2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>L1 no RT</td>
</tr>
<tr>
<td>2</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>B-actin</td>
<td>L2 no RT</td>
</tr>
<tr>
<td>3</td>
<td>BLANK</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NTC</td>
<td>N1 no RT</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yv7 +ve</td>
<td>N2 no RT</td>
</tr>
<tr>
<td>5</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>Ma1 no RT</td>
</tr>
<tr>
<td>6</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>Ma2 no RT</td>
</tr>
<tr>
<td>7</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>F1 no RT</td>
</tr>
<tr>
<td>8</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>MRP 1</td>
<td>F2 no RT</td>
</tr>
</tbody>
</table>
For some low abundance transcripts the template DNA amount was increased to 4µL. When a cDNA sample was assayed for the first time, a no-RT control containing RNA as template was used to confirm that co-amplification of genomic DNA was not adversely contributing to the results. Cycling conditions were as previously described (section 6.2.3).

6.2.5 Data analysis

The threshold for Ct determination was set at the point where amplification was above the background, but below the plateau, and where slopes were parallel when the data was viewed in log-linear mode. When assessing data quality the following criteria were applied: a) Sigmoidal amplification curve with Cts ≤ 35; b) Melt curve analysis confirming product specificity and c) Replicate Cts within 0.5. Where samples did not meet these criteria or results were ambiguous, PCR was repeated using more template cDNA.

Relative quantification was used to estimate gene transcription data. In this method, gene transcription is calculated by comparison to a reference, or calibrator gene. In the first set of analyses, ratios for relative transcription of the target gene compared to the β-actin reference gene were calculated. This was used to compare relative abundance of target genes and to compare transcription levels genes across life stages. The following formula was used:

\[
\text{Gene:Reference ratio} = \frac{1/E_{\text{target}}^{\text{Ct target}}}{1/E_{\beta\text{-actin}}^{\text{Ct β-actin}}}
\]

Where \(E\) = PCR efficiency, \(Ct\) = cycle threshold

For comparison of transcription between ivermectin exposed and untreated samples, the formula published by Pfaffl (2001) was used:

\[
\text{Fold change} = \frac{E_{\text{target}}^{\Delta \text{Ct target}}}{E_{\beta\text{-actin}}^{\Delta \text{Ct β-actin}}}
\]

Where \(\Delta \text{Ct} = \text{Ct untreated} - \text{Ct ivermectin treated.}\)
The Pfaffl formula is only suitable for comparing single experimental and control samples. However, in most studies, group wise comparisons are performed. To determine changes in relative transcription between multiple samples, two different approaches were employed. Initially, the equation above was applied to every possible sample combination between groups (using Microsoft excel). The values generated were exported to Prism V3.0.2 (GraphPad software Inc) to calculate median relative transcription, standard error and confidence intervals.

Statistical evaluation of real-time PCR data is not straightforward because data is derived from ratios and variation can be high. Normal distributions are not usually obtained and therefore traditional parametric tests cannot be readily applied. To overcome this, REST2005® (Relative Expression Software Tool) was used to further analyse the data and determine statistical significance (available at http://www.gene-quantification.info/). REST® is an automated program that enables group wise comparisons based on the Pfaffl calculation. Statistical analysis is done using the Pairwise Fixed Reallocation Randomization Test®. Randomization tests make no assumptions regarding distribution of data, and work by repeatedly and randomly allocating values between the two groups, noting the change in transcription ratio each time, for up to 50,000 iterations. The program also calculates levels of variation within the reference gene, thus checking its suitability for normalisation (Pfaffl et al., 2002).

6.3 Results
6.3.1 General comments and PCR reproducibility
PCR efficiency was calculated for all primer sets, and were in acceptable ranges of 92-104%. The results from diluted plasmid DNA templates also attested to the high sensitivity of the real-time PCR, with amplification occurring down to a concentration of 0.003 fg in some plasmids (data not shown). Inter and intra assay reproducibility was high, as demonstrated by replicate quality and comparison of the β-actin and Yv7 control Cts across multiple runs. Primer dimer formation and amplification of non-specific products was an early concern for some of the particularly low abundance genes. Specificity was improved by: a) Including a 70°C
inactivation step at the end of reverse transcription, which reduced non-specific PCR amplification; and b) Acquiring data at a higher temperatures (76-78°C) meant that fluorescence data represented only the specific product, as non-specific products and primer dimers melted below this temperature.

Statistical comparison of β-actin transcription between groups using REST did not show significant variation, attesting to its suitability for normalization in these experiments. Including β-actin in each run also allowed assessment of inter-assay variability. When the β-actin Cts of a single sample were compared across runs, on average less that 0.5 variation in Ct was observed (data not shown). Moreover, when RNA extracts were processed in two independent batches, most Cts obtained were comparable, showing that the RT step was also highly reproducible.

### 6.3.2 Overall transcription levels and life stage comparisons

Both the mu and delta classes of GST were highly expressed at all developmental stages of *S. scabiei*. Conversely, transcription of *SsCl* and ABC transporter genes was very low, representing less than 1% of β-actin abundance (Figure 6.2). Because of this, data could not be generated for larval stages of most genes, probably due to insufficient RNA template. When mean transcription of the ABC transporters were compared in adult mites, MRP1 appeared to be the least abundant (Figure 6.2).

For many genes, variability was observed in the data, particularly in the low abundance transcripts. Median transcription levels were however, consistent between life stages for most genes. Higher transcription of the mu class GST1 was seen in larva and females than for males and nymphs, and GST2 (delta class), MRP2 and MRP4 appeared to be up regulated in adult mites respective to juveniles. Conversely, P-glycoprotein and MRP1 were expressed more highly in juvenile stages (Table 6.4, Figure 6.3). Of note, these “basic transcription” values did not differentiate between ivermectin exposure status.
Figure 6.2: Relative transcription of GSTs, SsCl and ABC transporter genes in adult male and female *S. scabiei*.

Median relative expression was determined by comparison of transcription to β-actin. Error bars indicate interquartile ranges (25%-75% percentiles).
Table 6.4: Stage-specific gene transcription, relative to β-actin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Life Stagea</th>
<th>nb</th>
<th>Median transcription relative to β-actin</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST 1</td>
<td>L</td>
<td>9</td>
<td>0.63</td>
<td>0.18-1.16</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>11</td>
<td>0.22</td>
<td>0.16-0.51</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>16</td>
<td>0.39</td>
<td>0.33-0.59</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>11</td>
<td>0.69</td>
<td>0.21-2.49</td>
</tr>
<tr>
<td></td>
<td>Mix</td>
<td>11</td>
<td>0.19</td>
<td>0.12-0.42</td>
</tr>
<tr>
<td>GST 2</td>
<td>L</td>
<td>7</td>
<td>0.28</td>
<td>0.13-0.81</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>11</td>
<td>0.32</td>
<td>0.15-1.04</td>
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<tr>
<td></td>
<td>♂</td>
<td>16</td>
<td>0.60</td>
<td>0.47-0.99</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>11</td>
<td>1.26</td>
<td>0.08-5.29</td>
</tr>
<tr>
<td></td>
<td>Mix</td>
<td>11</td>
<td>0.18</td>
<td>-0.05-0.69</td>
</tr>
<tr>
<td>SsCl</td>
<td>L</td>
<td>n/a</td>
<td>0.0014</td>
<td>-0.0007-0.008</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>6</td>
<td>0.0024</td>
<td>0.001-0.17</td>
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<tr>
<td></td>
<td>♂</td>
<td>12</td>
<td>0.0024</td>
<td>-0.008-0.028</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>10</td>
<td>0.0023</td>
<td>0.0002-0.0087</td>
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<tr>
<td>Pgp</td>
<td>L</td>
<td>7</td>
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<td>0.0028-0.015</td>
</tr>
<tr>
<td></td>
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<td>0.002-0.074</td>
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<tr>
<td></td>
<td>♂</td>
<td>14</td>
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<td>0.001-0.016</td>
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<tr>
<td></td>
<td>♀</td>
<td>11</td>
<td>0.005</td>
<td>0.0001-0.0062</td>
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<tr>
<td></td>
<td>Mix</td>
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<td>-0.003-0.03</td>
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<td>0.0002-0.005</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>11</td>
<td>0.0004</td>
<td>-0.003-0.0045</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>9</td>
<td>0.0004</td>
<td>-0.002-0.008</td>
</tr>
<tr>
<td>MRP 2</td>
<td>L</td>
<td>n/a</td>
<td>0.0014</td>
<td>-0.007-0.028</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>6</td>
<td>0.0089</td>
<td>0.006-0.015</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>13</td>
<td>0.0053</td>
<td>-0.001-0.023</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>8</td>
<td>0.0019</td>
<td>0.0005-0.008</td>
</tr>
<tr>
<td>MRP 3</td>
<td>L</td>
<td>n/a</td>
<td>0.0057</td>
<td>0.0024-0.015</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>10</td>
<td>0.0060</td>
<td>0.002-0.044</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>14</td>
<td>0.0037</td>
<td>-0.0046-0.029</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>8</td>
<td>0.0015</td>
<td>-0.006-0.018</td>
</tr>
<tr>
<td>MRP 4</td>
<td>L</td>
<td>n/a</td>
<td>0.0001</td>
<td>-0.0013-0.0023</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>3</td>
<td>0.0017</td>
<td>-0.0033-0.023</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>7</td>
<td>0.0066</td>
<td>-0.0065-0.03</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>6</td>
<td>0.001</td>
<td>-0.01-0.033</td>
</tr>
</tbody>
</table>

a: L= larvae, N= nymph, ♂= adult male, ♀= adult female; b: 'n'= pooled mite sample as detailed in Table 6.1; n/a= PCR failures, no data available
Figure 6.3: Life-stage specific transcription of S. scabiei GSTs, SsCl and ABC transporter genes relative to β-actin.
Scatter plot showing individual samples, with median observations for each life stage indicated by the horizontal line.
6.3.3 Transcription in ivermectin exposed mites

Data for adult females, adult males and mixed mite preparation were combined and analysed for overall changes in transcription correlated with ivermectin exposure. Juvenile stages were excluded, as one cannot precisely determine age with relation to length of drug exposure. For GST1 and MRP1, no changes were observed in ivermectin-exposed mites. Pgp, MRP2 and MRP3 appeared to be down regulated, but the P-values associated with these observations were very high. The largest changes were in GST2, SsCl, and most strikingly, MRP4. Of these only MRP4 reached statistical significance, with a median 6-fold up regulation in ivermectin exposed mites. GST2 and SsCl were upregulated by 2.6 and 2.3 fold respectively (Table 6.5, Figure 6.4)

In the next set of analyses, data were separated according to life stage and ivermectin exposure, to see if trends were specific to a particular life stage. GST2 and MRP4 were up regulated in all life stages, but this was more dramatic in female mites compared to males. In MRP4, the mixed life stages were also strongly up regulated (Table 6.6, Figure 6.5). When results for SsCl were broken down, the data was ambiguous, with increased transcription observed in mixed mite preparations but not in males or females. This suggests that up regulation may be attributed to ivermectin exposed juvenile mites. When nymphs were compared, median up regulation in the ivermectin exposed group was 4-fold (Figure 6.5).
Table 6.5: Transcription in ivermectin exposed adult mites relative to untreated controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Median fold up-regulation</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST1</td>
<td>1.171</td>
<td>0 - 808.02</td>
<td>0.858</td>
</tr>
<tr>
<td>GST2</td>
<td>2.636</td>
<td>0.01 - 753.90</td>
<td>0.177</td>
</tr>
<tr>
<td>SsCl</td>
<td>2.289</td>
<td>0.05 - 85.84</td>
<td>0.120</td>
</tr>
<tr>
<td>Pgp</td>
<td>0.437</td>
<td>0.03 - 7</td>
<td>0.345</td>
</tr>
<tr>
<td>MRP1</td>
<td>1.098</td>
<td>0.015 - 28.990</td>
<td>0.951</td>
</tr>
<tr>
<td>MRP2</td>
<td>0.627</td>
<td>0.018 - 12.720</td>
<td>0.968</td>
</tr>
<tr>
<td>MRP3</td>
<td>0.199</td>
<td>0.004 - 5.638</td>
<td>0.173</td>
</tr>
<tr>
<td>MRP4</td>
<td>6.252</td>
<td>0.147 - 369.867</td>
<td>0.028*</td>
</tr>
</tbody>
</table>

Figure 6.4: Fold changes in gene transcription in ivermectin exposed adult mites.

Median transcription, relative to untreated controls and normalised to β-actin.
Table 6.6: Life stage specific post IVM transcription

<table>
<thead>
<tr>
<th></th>
<th>Adult males</th>
<th></th>
<th>Adult females</th>
<th></th>
<th>Mixed life stages</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median transcription</td>
<td>95% CI</td>
<td>P</td>
<td>Median transcription</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>GST1</td>
<td>1.48</td>
<td>1.57-2.27</td>
<td>0.755</td>
<td>0.73</td>
<td>0.76-1.48</td>
<td>0.789</td>
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<tr>
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<td>1.59</td>
<td>1.79-2.64</td>
<td>0.612</td>
<td>5.08</td>
<td>4.99 – 17.56</td>
<td>0.332</td>
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<tr>
<td>SsCl</td>
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<td>0.06 - 11.21</td>
<td>0.587</td>
<td>1.22</td>
<td>0.12 - 17.79</td>
<td>0.942</td>
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<td>Pgp</td>
<td>1.54</td>
<td>1.5-3.11</td>
<td>0.576</td>
<td>0.18</td>
<td>0.21 – 1.82</td>
<td>0.802</td>
</tr>
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<td>MRP1</td>
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<td>0.01-16.79</td>
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<td>3.29</td>
<td>1.9-49.04</td>
<td>0.375</td>
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<td>0.5-0.96</td>
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<td>MRP3</td>
<td>0.29</td>
<td>0.01-5.53</td>
<td>0.099</td>
<td>0.15</td>
<td>0.03 - 2.18</td>
<td>0.676</td>
</tr>
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<td>MRP4</td>
<td>2.07</td>
<td>0-21.82</td>
<td>0.429</td>
<td>8.56</td>
<td>0-222.9</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Figure 6.5: Life stage specific post ivermectin transcription.
Scatter plot shows each sample comparison, with median observations indicated by the horizontal line.
6.4 Discussion

Preliminary transcription studies on candidate ivermectin resistance genes from *S. scabiei* were conducted. These molecules represented the three major mechanisms potentially involved in ivermectin resistance, being: a) target-site alteration; b) metabolic detoxification; and c) drug efflux. To our knowledge, these studies represent the first application of qRT-PCR to scabies mite research.

Sound experimental design is critical for making valid interpretations in qRT-PCR. Although difficulties were encountered when working with these microscopic mites, consideration was given to standardising the assays as much as possible. The main issue was working with tiny quantities of RNA, making assessment problematic. We found that quantitating samples using conventional spectrophotometry did not provide adequate sensitivity or accuracy, and denaturing agarose gel electrophoresis requires even larger amounts of RNA which were unavailable. However, recently developed RNA quantification tools such as the NanoDrop spectrophotometer, Agilent Bioanalyser and RiboGreen fluorometric assays promise orders of magnitude more sensitivity, and may be a valuable addition to future studies. Although these RNA samples could not be quantified, using relative quantification methods meant that equalizing starting amounts of cDNA was not imperative, so long as reference gene expression was included in each assay (Wong and Medrano, 2005). Moreover, studies found that using vastly different cDNA template concentrations did not influence relative expression ratios obtained (Pfaffl *et al.*, 2002).

*S. scabiei* β-actin was used as the reference gene for normalization purposes. The ideal reference is expressed equally in all samples regardless of experimental conditions. It has been found that even the most commonly used normalising genes can vary to some extent (Perez-Novo *et al.*, 2005), and thus it is important to validate that the chosen reference does not change between developmental stages or experimental conditions. Ideally, normalization should be carried out using multiple reference genes (Nolan *et al.*, 2006). In our situation, with RNA samples extremely limited, this was not possible. Results did however indicate that β-actin was a suitable reference gene for these experiments.
We determined the transcription levels of two *S. scabiei* glutathione S-transferases, GST1 & 2, belonging to the mu and delta classes respectively. Both transcripts were expressed in relative abundance, which is not surprising given their ubiquity in other eukaryotes. Comparison between life stages showed that GST1 was expressed most highly in larvae and adult female mites, whereas GST2 was more abundant in adult mites of both sexes. Other studies on acari report similar developmental regulation of GST, with a mu class GST highly expressed in larval stages of cattle ticks (*Boophilus microplus*) (He et al., 1999a). GST levels were compared in ivermectin-exposed mites relative to untreated controls. No changes were observed in GST1, but GST2 transcription was up-regulated by a median of 2.6-fold. This was most evident in adult female mites, with 5-fold over transcription. Although these trends were clear, they did not reach statistical significance.

In arthropods, increased activity of delta and epsilon class GSTs has been associated with resistance to organophosphates, DDT and pyrethroids (reviewed in Hemingway et al., 2004). Significantly, GST activity has been linked to abamectin resistance in two-spotted spider mites (*Tetranychus urticae*) (Campos et al., 1996) (Stumph and Nauen, 2002), although the specific class/es responsible have not been defined. In *B. microplus*, recombinant GST activity was not modulated by ivermectin (da Silva Vaz et al., 2004), and transcription patterns did not change between untreated and organophosphate resistant strains (He et al., 1999a). However, these reports focused on a single mu class, whereas GSTs implicated in insecticide resistance to date belong to either delta or epsilon classes (Ranson et al., 2002). Considering this, and in light of our present findings, mu GSTs may not be of primary importance, therefore it might be more appropriate for future studies to concentrate on classes already demonstrated to confer drug resistance.

We also investigated the transcription levels of SsCl, a pH-gated chloride channel. SsCl was expressed at similar levels between juvenile and adult mites, although larval levels could not be assessed due to insufficient RNA. There was some evidence of SsCl up-regulation in ivermectin-exposed mites, but like GST, the change was modest and did not reach statistical significance. Although yet to be confirmed, preliminary studies on this channel suggest an interaction with ivermectin (chapter 5). If SsCl is indeed a physiological target for ivermectin in *S. scabiei*, up-
regulation may translate to an increased number of binding sites and subsequent reduction in ivermectin toxicity. Such a mechanism has been proposed to mediate laboratory selected abamectin resistance in the housefly *Musca domestica* (Konno and Scott, 1991), although there have been no molecular studies to confirm this. Interestingly, over expression was only observed in the nymph and mixed life-stage samples. This suggests that SsCl up-regulation may be specific to ivermectin-exposed juvenile *S. scabiei*. This will need to be investigated more closely using larger mite numbers, and under conditions of controlled ivermectin exposure if possible.

Finally, we compared transcription levels in five ABC transporter genes: P-glycoprotein and four multidrug resistance associated proteins. When compared to β-actin and GSTs, these transcripts are in very low abundance. Nonetheless, transcription could be detected in all life-stages for most genes, although MRP transcription in larval stages was too slight to be quantified accurately. Again, this probably relates more to lack of RNA template rather than specific larval down-regulation. There was little meaningful difference in transcription between developmental stages, although at such low levels any changes may be difficult to detect with accuracy. Similarly low ABC transporter transcription has been found in other organisms, and it has been suggested that these genes may be virtually “silent” in parasitic nematodes until true drug resistance emerges (A. Roulet, pers. comm.).

MRP4 transcription was significantly correlated with ivermectin exposure, with a median 6-fold up-regulation. This was observed in all life stages, although like GST2, was most striking in female mites. This is the first molecular evidence that MRPs may be involved in the development of ivermectin resistance. Recent reports have demonstrated that ivermectin can act as a substrate for MRPs (Lespine et al., 2005), and Ardelli & Prichard (2004) found ivermectin selection on an MRP like transporter from *O. volvulus*. It will be interesting to see whether future studies on ivermectin resistant nematodes report similar MRP up-regulation. Notably, the pattern of MRP4 and GST2 up-regulation was similar. MRPs are known to be glutathione conjugate transporters, so the concept of these two molecules working together in the detoxification and extrusion of ivermectin is intriguing. However, much further work is required before such speculation is warranted.
Although efforts were made to control and monitor for assay variation, there were major differences in the results obtained for some genes, highlighted by wide confidence intervals. This probably explains why despite clear trends in the data, only one (MRP4) was evaluated by REST to be statistically significant. When using relative expression ratios, a high degree of variability in results obtained is not unusual (Pfaffl et al., 2002). It is also important to consider transcription ratios within their biological context. For example, a 2-fold up-regulation of the already highly expressed GST2 may be more relevant than a 6-fold up-regulation of the low abundance MRP4, despite the statistics.

The variability we observed may be attributed to several reasons. Firstly, statistical insignificance of data may have occurred due to the relatively low sample sizes between the groups, and the subsequent difficulty in identifying potential outliers with confidence. This was compounded by very low transcription levels of ABC transporters and SsCl, with most Cts in the 28-33 range. At these levels, the PCR efficiency would be approaching its limit of linearity, with a reduction in sensitivity leading to an inevitable increase in variation. Future studies must focus on increasing sensitivity for these low abundance transcripts. Improvements may include increasing RNA template by using more mites, and narrowing focus to fewer genes of interest (thereby increasing the amount of cDNA template available). Some studies report that RT sensitivity is improved by priming with random 15mers (Nolan et al., 2006) (this study used nonamers). Additionally, using a probe based detection system instead of SYBR green may provide more sensitivity for low abundance targets (Qiagen Research, 2004) although for these preliminary investigations SYBR was the most convenient and cost effective option.

Variability may also have been largely due to genetic characteristics of the samples themselves. It is important to remember that mites were collected from a clinical setting, and were not exposed to long-term ivermectin selection. The mites in any pooled sample therefore represent a genetically heterogeneous host derived population, with a variety of responses to drug exposure. Additionally, it is difficult to determine whether all mites have been exposed to the same levels of ivermectin in vivo, which may also contribute to variability of drug responses in individual mites.
If one were to compare these results to those from a stronger drug selected population, the data would almost certainly be tighter.

Clinically, all patients in this particular study responded adequately to combined benzyl benzoate and ivermectin treatment, and in vitro sensitivities (Chapter 3) did not indicate resistance was established in these populations. Therefore, the changes apparent after treatment may represent innate mechanisms to ivermectin exposure. A difficulty when working within the clinical environment is the potential confounding influence of other acaricides co-administered with ivermectin. For this study, all mites collected were cross-referenced to the treatment history of the patient. Most mites in the ivermectin-exposed group were collected prior to the commencement of topical therapy, although some were also exposed to benzyl benzoate. An important aspect of future work will be to compare the effects of different treatment strategies on transcription levels, although this would require large sample numbers and careful co-ordination with the treating clinicians. Another possibility would be to examine mites collected over a time course of in vitro drug exposure. Thus all mites would receive equal levels of exposure in a controlled manner. A limitation to this however is the inability to maintain mites away from the host.

The fact we observed transcription changes after relatively short ivermectin exposure suggests even more dramatic changes would be evident with increasing drug pressure. Therefore, our concerns regarding the rapid emergence of ivermectin resistance may be justified. The potential for rapid selection even in the absence of prolonged drug pressure was highlighted by recent findings of intra-host copy number amplification of the P-glycoprotein homologue pfmdr1 in patients following antimalarial treatment (Uhlemann et al., 2007). Significantly, recent drug sensitivity assays performed on mites from a severely infested crusted scabies patient showed decreasing in vitro sensitivity over the course of ivermectin treatment (Chapter 3). A priority will be to apply this newly developed qRT-PCR assay to these mite samples to determine whether the in vitro data is correlated with the transcription changes reported herein.

Presently, drug resistance studies on *S. scabiei* are challenging due to the lack of in vitro culture system or availability of animal models. We are restricted to working within the clinical environment and are unable to select for resistance in the
laboratory, which is fundamental to most drug resistance studies. In collaboration with the Queensland Institute of Medical Research malaria and scabies laboratory, we are working towards development of an animal model for scabies, which would greatly enhance the present work. By closely monitoring transcription changes over the course of drug selection, great insights may be obtained regarding the emergence of resistance.

For future studies, it will be important to further investigate and define the mechanisms of MRP4 and GST2 over-expression. For example, MRP amplification may possibly be conferred by increases in gene copy number, as observed in malaria parasites (Price et al., 2004), or perhaps by changes to gene promoter activity. Likewise, GST over expression may be mediated by mutations in regulatory regions resulting in increased enzyme activity (Ding et al., 2003). It will also be interesting to see whether additional S. scabiei delta-GSTs are also associated with drug resistance. Proposed biochemical assays using whole-mite extracts and recombinant GST2 will enable us to determine whether the observed GST up-regulation is evident at the enzymatic level.

For the first time, a quantitative RT-PCR assay specific to S. scabiei has been developed. This method should prove useful for further research on scabies mite biology. We demonstrated that several proteins might be implicated in the emergence of ivermectin resistance in scabies. In particular, the involvement of GST2 and MRP4 highlights a previously unexplored mechanism of resistance, which may have broader implications for research concerning ivermectin resistance in other parasites.
CHAPTER 7

Chapter 7

Genetic polymorphisms in candidate ivermectin resistance genes from *Sarcoptes scabiei*

7.1 Introduction

In eukaryotes, the development of drug resistance generally occurs through selection for existing alleles in a population, rather than the development of de-novo mutations. Resistance-associated alleles are most likely present in a population at low frequencies prior to treatment, with subsequent drug pressure selecting for these alleles and causing an alteration in genotypic frequencies (Prichard, 2001). These genetic changes precede the manifestation of clinical resistance, by which time resistance alleles are already in high frequency (Wolstenholme *et al.*, 2004). The application of molecular techniques to monitor populations at the genotypic level is therefore useful to detect these early changes before resistance becomes widespread and beyond management.

Such approaches have been widely applied to investigate the emergence of ivermectin resistance in parasitic nematodes such as *Haemonchus contortus*, *Onchocerca volvulus* and *Cooperia oncophora*. Subsequently, several genes were recognised to be under selection from ivermectin. These include P-glycoprotein and other ABC transporters (Ardelli *et al.*, 2005a; Ardelli *et al.*, 2005b; Ardelli *et al.*, 2006; Ardelli and Prichard, 2004; Blackhall *et al.*, 1998a; Eng and Prichard, 2005; Sangster *et al.*, 1999; Xu *et al.*, 1998); β-tubulin (Eng *et al.*, 2006; Eng and Prichard, 2005); Glutamate-gated chloride channels (Blackhall *et al.*, 1998b; Njue and Prichard, 2004); and a GABA-gated chloride channel (Blackhall *et al.*, 2003). Although no explicit association of these polymorphisms with ivermectin resistance has been demonstrated for most of these, they nonetheless show that ivermectin treatment is changing the allelic frequencies of these genes, and they may prove useful markers in monitoring the development of drug resistance.
The capability for an organism to acquire drug resistance is thought to be associated with the level of genetic diversity within a population, as high diversity increases the probability of pre-existing resistance alleles. *H. contortus* for example is extremely genetically diverse (Prichard, 2001), with ivermectin resistance developing very rapidly in both field and laboratory settings (Coles *et al.*, 2005; Shoop, 1993). In contrast, relatively little is known about genetic diversity of *S. scabiei*. Analysis of mitochondrial and microsatellite markers indicate a high degree of genetic polymorphism, with up to 46 alleles at a particular microsatellite locus reported in a single population of mites (Walton *et al.*, 1999b), and an average of 12 alleles per microsatellite loci (Walton *et al.*, 2004a). However mitochondrial and microsatellite DNA are known to have high mutation rates (Graur and Li, 2000), and to date no investigations have been undertaken on other nuclear genes or those potentially under drug selection.

Single-strand conformation polymorphism (SSCP) analysis is a useful way for screening unknown populations for genetic variability (Orita *et al.*, 1989a). It is based on the principle that under non-denaturing conditions, a single strand of DNA will adopt a conformation dependant on its sequence, with even single base changes changing conformation sufficiently to be detected as a mobility shift on a polyacrylamide gel (Humphries *et al.*, 1997). It allows PCR products to be screened rapidly and inexpensively for sequence variation, and is generally more sensitive than other genotyping methods such as restriction fragment length polymorphism (RFLP) analysis, but less expensive and time consuming that direct sequencing. Once a polymorphism has been identified via SSCP, direct sequencing can then be used to pinpoint the specific sequence alteration/s.

Described in this chapter is a pilot study, whereby SSCP was applied to individual *S. scabiei* mites collected from crusted scabies patients with documented in vitro and/or clinical ivermectin sensitivity profiles, in an attempt to investigate the following questions:

1. What are the levels of heterogeneity in genes of interest to ivermectin resistance in *S. scabiei*? Which alleles are most common?
2. Are any trends in allelic frequencies consistent with a) different years of collection; b) different crusted scabies patients or c) sub optimal responses and / or in vitro ivermectin sensitivity profiles?

7.2 Methods

7.2.1 Mites

*S. scabiei* var. *hominis* mites were collected from crusted scabies patients presenting to Royal Darwin Hospital (chapter 2). For these preliminary studies, a total of 57 individual mites were genotyped. Mites were selected from database records according to the following in vitro survival times to ivermectin: sensitive (60 minutes or less); moderately sensitive (120-270 minutes); or resistant (1290 minutes or greater). Additional mites were selected from a patient in January 2000 with documented clinical ivermectin resistance (chapter 3) (Currie *et al.*, 2004). Although these additional mites did not have in vitro sensitivities performed, they were collected from the same patient and time as the in vitro “resistant” mites above. Overall years sampled ranged from 1999 to 2006, with most mites collected in 2000. It is important to note that due to laboratory logistics not all genes were analysed for all mites (Table 7.1,Table 7.2). DNA from individual mites was prepared as described in chapter 2.

7.2.2 Genes analysed

Four *S. scabiei* genes were examined (Table 7.3):

a) β-tubulin- region corresponding to an area identified as being under strong ivermectin selection in parasitic nematodes (Eng *et al.*, 2006; Eng and Prichard, 2005).

b) P-glycoprotein- region in C-terminal ATP-binding domain, downstream of Walker B motif (chapter 4)

c) Multidrug resistance protein 3- cytoplasmic linker region (chapter 4)

d) pH-gated chloride channel (SsCl, chapter 5), in which three regions were surveyed. Fragments 1 & 2 were located in the N-terminal extracellular ligand-
binding domain, and fragment 3 was in the TM 2-3 region of the ion-channel domain.

Table 7.1: *S. scabiei* mites used for analysis of $\beta$-tubulin, Pgp, MRP3 & SsCl fragment 3

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Collection date</th>
<th>In vitro survival (min)</th>
<th>Patient code</th>
<th>Collection date</th>
<th>In vitro survival (min)</th>
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<td>WB</td>
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<td>60</td>
<td>17</td>
<td>MC</td>
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<tr>
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<td>WB</td>
<td>02/2004</td>
<td>60</td>
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<td>WB</td>
</tr>
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Table 7.2: *S. scabiei* mites used for analysis of $\beta$-tubulin and SsCl fragments 1 & 2

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<td>30</td>
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<td>TB</td>
<td>10/02</td>
<td>35</td>
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</tr>
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<td>TB</td>
<td>10/02</td>
<td>35</td>
<td>19M</td>
<td>WB</td>
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<tr>
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<td>35</td>
<td>20M</td>
<td>WB</td>
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<td>35</td>
<td>21R</td>
<td>MC</td>
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<td>11/02</td>
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<td>MC</td>
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<td>MS</td>
<td>02/03</td>
<td>45</td>
<td>23R</td>
<td>MC</td>
</tr>
<tr>
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<td>MC</td>
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<td>MC</td>
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<td>06/00</td>
<td>120</td>
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<td>120</td>
<td>28R</td>
<td>MC</td>
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<tr>
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<td>WB</td>
<td>02/04</td>
<td>120</td>
<td>29R</td>
<td>MC</td>
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<tr>
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<td>MC</td>
<td>02/04</td>
<td>120</td>
<td>30R</td>
<td>MC</td>
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Table 7.3: Gene fragments analysed by SSCP with primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size</th>
<th>cDNA region</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>Temp b</th>
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<tbody>
<tr>
<td>β-tubulin</td>
<td>340bp</td>
<td>372-712</td>
<td>5914F</td>
<td>GAT GTG GTC CGA AAA GAA GC</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5914R</td>
<td>CCG AGA CCA AAT GAT TGA GA</td>
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<tr>
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<td>155bp</td>
<td>718-873</td>
<td>236F2</td>
<td>AGG CAA CTT CAG CAC TCG AT</td>
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</tr>
<tr>
<td>MRP 3</td>
<td>219bp</td>
<td>248-467</td>
<td>C03F3</td>
<td>CAC CCA ATC CCA TAA GAA TGA</td>
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<td></td>
<td></td>
<td>C03R3</td>
<td>TGA TGA CCG TTT TCG TAG GG</td>
<td></td>
</tr>
<tr>
<td>SsCl</td>
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<td>83-360a</td>
<td>B08F6</td>
<td>ACAATGTCATCATTGAGACAT</td>
<td>60</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>B08R9</td>
<td>CGTGAGACACGTTAATCTGGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2) 294bp</td>
<td>425-648a</td>
<td>B08F5</td>
<td>GTG ATG GAC ATG TTC GAA TGA G</td>
<td>54</td>
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<td></td>
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<td>B08R1</td>
<td>TTC TGA TAG ACC GAA TAG CC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3) 329bp</td>
<td>596-925a</td>
<td>B08F3</td>
<td>TGA TTT CTA TAT GTC GGG CCA TTT G</td>
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<td></td>
<td></td>
<td>B08R6</td>
<td>CAG CGA ACC AAA GAT CAA CA</td>
<td></td>
</tr>
</tbody>
</table>

a introns present in these regions; b PCR annealing temperature

7.2.3 PCR & SSCP

PCR components were standard (chapter 2), except for SsCl fragments 1 & 2, where MgCl₂ was added to a final concentration of 1.75mM. Cycling conditions were: initial denaturation, 95°C, 2 min; 94°C, 30s; 54-60°C, 30s; 72°C, 30s for 40 cycles, followed by final extension of 72°C, 5 min. Successful amplification was confirmed by 1.5% agarose gel electrophoresis.

1-3 µL of the PCR product was mixed with SSCP loading buffer (95% v/v formamide, 10mM NaOH, 0.25% v/v bromophenol blue, and 0.25% v/v xylene cyanole) at a dye: product ratio of 15:1. Products were denatured at 95°C for 5 min and immediately cooled on ice before loading onto a 10%-14% 49:1 or 37.5:1 non-denaturing polyacrylamide gel (depending on the genes analysed). Electrophoresis was performed at 110V (5-10W) for 20-28 hours at room temperature. Gels were stained with 0.5 µg/mL ethidium bromide and viewed on a transilluminator. SSCP polymorphs were assigned according to the most commonly occurring fragment patterns.
7.2.4 Sequencing of SSCP polymorphs

Selected PCR products representing SSCP variants for SsCl fragments 1 & 2 were purified and sequenced in both directions with the corresponding gene-specific primers. The edited sequences were then aligned with the existing cDNA and genomic DNA reference sequences (chapter 5) using ‘Blast2 sequences’ (Tatusova and Madden, 1999) to identify sequence variants.

7.3 Results

57 mites were surveyed for β-tubulin, with two polymorphs identified (Figure 7.1). Most mites possessed polymorph A, with only three mites showing polymorph B. Interestingly, these mites (23, 26 and 27) had documented in vitro ivermectin resistance (Table 7.1). However, this variant was not observed in additional mites collected from this patient admission (Table 7.2).

No SSCP variants were observed in the 27 mites assessed for P-glycoprotein, MRP3 and SsCl Fragment 3 (Figure 7.2).

For SsCl fragment 1, six SSCP polymorphs were identified in the 29 mites tested (Figure 7.3a), with Polymorph B the most frequently occurring. Polymorph C was only found in mites with moderate ivermectin sensitivity. The remaining alleles were present in low frequencies, with polymorph A only seen in sensitive mites, while D, E & F were observed in clinically resistant mites (Figure 7.3b). These polymorphs were sequenced and correlated to six sequence types. Variations were identified consistently at four positions (Table 7.4) At position 100, chromatograms for polymorphs A & C were indicative of T/G heterozygote. No mites homozygous for T at this position were seen, probably because this introduces a stop codon and would result in non-functional protein. A synonymous A111T SNP was observed in polymorph C. Polymorphs D and E showed an A249G SNP, while polymorph C was heterozygous at this site. This SNP was located within an intron and therefore did not alter the coding sequence. Comparison with the reference genomic DNA sequence showed an A-T SNP.
Figure 7.1: β-tubulin polymorphs.
a) Representative SSCP patterns, b) Polymorph frequencies of β-tubulin alleles. Mites were grouped according to in vitro/clinical ivermectin sensitivity (sensitive: survival <60 minutes, moderate: survival 120-170 minutes, resistant: survival >1290 minutes OR sub-optimal clinical response)

Figure 7.2: Representative SSCP patterns for P-glycoprotein, MRP3 and SsCl fragment 3.
No polymorphisms were identified in these genetic regions.
at position 305 for all polymorphs. This is also apparent in the cDNA sequence, suggesting that in this case the genomic reference sequence is mutated or incorrect. Polymorphs B, D & E were identical on the basis of these four sites. However, additional sequence variants were observed in polymorphs B & D. Polymorph B had a T-A transversion at position 191, and A-T insertion at position 195 (both these were located within the intron). Over the first 100 nucleotides of polymorph D double peaks were frequently observed in the chromatograph, making it difficult to call bases with confidence. Although polymorph D is clearly a different sequence type, this sample will need to be cloned to clearly resolve the sequence differences.

Three SSCP polymorphs were observed for SsCl Fragment 2 in the 23 mites successfully amplified (Figure 7.4a). Mites in the ‘moderate’ category all corresponded to polymorph A. Polymorphs A and B were most common in sensitive mites, and resistant mites were the most variable, with all three polymorphs observed (Figure 7.4b). Representative polymorphs were sequenced, and several SNPs identified. However, these variations were only seen in the intron region, and thus did not result in coding changes to the protein (Figure 7.4c).
Figure 7.3: SsCl fragment 1 polymorphs.
(a) Representative SSCP patterns, b) Allele frequencies.

Table 7.4: Single-nucleotide polymorphisms (SNPs) identified in SsCl Fragment 1

<table>
<thead>
<tr>
<th>Sequence</th>
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<th>111</th>
<th>249</th>
<th>305</th>
</tr>
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<tbody>
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<td>G</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>cDNA library</td>
<td>G</td>
<td>A</td>
<td>n/a*</td>
<td>T</td>
</tr>
<tr>
<td>A</td>
<td>T/G</td>
<td>A</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>B</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>T</td>
</tr>
<tr>
<td>C</td>
<td>T/G</td>
<td>T</td>
<td>A/G</td>
<td>T</td>
</tr>
<tr>
<td>D</td>
<td>G</td>
<td>A</td>
<td>G</td>
<td>T</td>
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<td>A</td>
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<tr>
<td>F</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>T</td>
</tr>
</tbody>
</table>

*Intronic region, i.e not present in cDNA
Figure 7.4: SsCl Fragment 2 polymorphs.
(a) Representative SSCP patterns
(b) Allele frequencies
(c) Alignment of sequence variants.
7.4 Discussion

This chapter describes initial investigations into the genetic diversity of four *S. scabiei* genes- two ABC transporters, β-tubulin and a pH-gated chloride channel. These molecules were selected due to their association with ivermectin resistance in other organisms. Mites surveyed represented a cross-section from several crusted scabies patients, multiple years, and most with characterised in vitro ivermectin sensitivity profiles. It is important to emphasise that with the low mite numbers sampled, it was not the intention to conduct a detailed investigation into population genetics. Instead, the objective of this work was to gather preliminary data regarding the degree of polymorphism in these genetic regions, and to evaluate the validity of these approaches for future studies.

The apparent lack of genetic heterogeneity observed in the two ABC transporters investigated (P-glycoprotein and MRP3) suggests they may not be involved in nor be useful indicators of ivermectin resistance in *S. scabiei*. The result with P-glycoprotein was somewhat unexpected, as the particular region examined was found to be very polymorphic in *O. volvulus*, with up to 10 alleles identified in ivermectin naïve worms (Ardelli *et al.*, 2005b). However, our result may be uninformative, given that only a small region of a relatively large (4.2kb) gene was analysed. Furthermore, this gene most likely represents one of several P-glycoproteins present in *S. scabiei*. In combination with the previous transcription data (chapter 6), this particular P-glycoprotein appears not to be involved in ivermectin resistance. Although it was of considerable interest, due to time limitations and initial PCR problems, MRP4, identified to be over-expressed in ivermectin exposed mites, was not investigated in the present study.

The results obtained from β-tubulin were inconclusive. An altered SSCP polymorph was observed in three mites with documented in vitro ivermectin tolerance. However, the remaining 13 “resistant” mites did not show this alteration, despite being collected from the same patient at the same time. The analysis was repeated several times, so the result cannot be attributed to experimental artefact. The SSCP pattern observed in the tolerant mites suggested transition from a heterozygous to homozygous genotype, although this was not resolved by sequence analysis. Further investigations of this apparent polymorphism in stronger drug selected populations...
would be beneficial, especially in light of recent evidence that *O. volvulus* and *H. contortus* β-tubulins are clearly under selection pressure from ivermectin (Eng *et al.*, 2006; Eng and Prichard, 2005). Although it is difficult to extrapolate the significance of these nematode findings to arthropods, the fact that we analysed the same genetic region, coupled with the high conservation of this gene suggests that any alterations may be of functional importance.

As mentioned previously, analysis of small fragments may not be sufficiently informative, especially if different regions within a gene are under different selection pressures or mutate at different rates (Graur and Li, 2000). To address this, a more comprehensive approach was taken with *SsCl*. We analysed regions previously associated with drug resistance, including the N-terminal ligand binding domain (fragments 1 & 2); and the TM2 region of the ion channel domain (fragment 3). Although no diversity was observed in fragment 3, the ligand-binding domain was quite polymorphic, given the small sample size.

The high degree of polymorphism in *SsCl*, particularly when compared to the conservation of the other genes analysed, suggests this region undergoes high mutation rates. Njue and Prichard (2004) observed similar heterogeneity in the ligand-binding domain of *C. oncophora* GluCl, identifying nine alleles within a 228bp region, with one of these mutations associated with altered channel kinetics (Njue *et al.*, 2004). Whether the heterogeneity we observed is due to random genetic drift or other selective pressures cannot be determined at this time. Notably, the fragments analysed contained highly repetitive regions, and such segments may be prone to higher mutation rates (Graur and Li, 2000). It is important to remember that genetic evidence of selection does not confirm the functional presence of resistance. However, given the degree of polymorphism, it is possible that under favourable conditions, a conformational altering mutation will be selected, if *SsCl* is indeed a physiological target for ivermectin as predicted (chapter 5).

Interestingly, mites collected from a patient with clinical ivermectin treatment failure were the most polymorphic for *SsCl*. This is at odds with the notion that drug resistance results in a reduction in genetic heterogeneity (Wolstenholme *et al.*, 2004). However, similar studies on LGICs in *H. contortus* and *C. oncophora* observed that although resistance was correlated with selection for a particular allele, there was no
discernable reduction in overall variability (Blackhall et al., 1998b; Njue and Prichard, 2004). The low numbers in this study, make it impossible to draw any conclusions linking allele frequencies to a resistance phenotype. Furthermore, most of our drug sensitivity assays are conducted on mites collected from patients prior to the commencement of treatment. Thus, the assay often represents the first exposure to the drug. Because genetic selection is a generational process, even with larger numbers, we may not see a statistically significant association of resistance phenotype with a particular allele, in the absence of previous selection pressure. Conversely, changes in allele frequencies may be detected if mite populations have been exposed to prolonged suboptimal treatment, or if infestation is due to recrudescence rather than re-infestation.

Although SSCP was applied successfully to identify genetic polymorphisms in scabies mites, there are several issues associated with this technique. There are many factors affecting the migration of single-stranded DNA, including temperature, acrylamide percentage, acrylamide:bisacrylamide ratio, DNA concentration, and the inclusion of additives such as glycerol (Humphries et al., 1997). The effect of sequence alterations on mobility is unpredictable (Orita et al., 1989b), and thus the ideal SSCP conditions must be determined empirically. This is particularly difficult if one is screening for unknown polymorphisms. In future, the application of high-throughput, automated approaches such as capillary electrophoresis (CE-SSCP) may help reduce the labour-intensive optimization process (Kozlowski and Krzyzosiak, 2001). Another consideration is that SSCP loses resolution with increasing fragment length. Most reports suggest a product size of 150-300bp for adequate sensitivity (Orita et al., 1989b) (Humphries et al., 1997), although products of up to 500bp have been genotyped successfully (J Eng, pers. comm.). Several of our fragments were between 300 and 400bp, so there is the slight possibility that not all polymorphisms present were detected.

A new alternative to SSCP is High Resolution Melting (HRM$^\text{TM}$) analysis (Wittwer et al., 2003). This is based on the principle that the melting properties of DNA are highly dependant on the nucleotide sequence. HRM$^\text{TM}$ uses ‘new generation’ fluorescent intercalating dyes in real-time PCR to allow melt analysis in increments as low as 0.1°C, to detect genotypes with high sensitivity
However, one limitation at present is that the melting properties of the sequence are influenced by the components of the PCR and template preparation (Corbett Research, 2006b); and different melting curves may be obtained for an identical template with different concentrations (personal observations). This may be problematic when using our relatively crude scabies mite DNA preparations. Nonetheless, with further research and optimization, HRM™ looks to be promising alternative to SSCP.

In summary, this work has given new insights into the genetic heterogeneity of *S. scabiei*. It is becoming increasingly likely that ivermectin will be incorporated into mass treatment programs for control of scabies in northern Australia. Given the genetic diversity observed in a putative ivermectin target, it is highly probable that resistance alleles already exist in these mite populations. Continued monitoring of the effects of drug selection on candidate resistance genes is paramount, particularly in the advent of a mass treatment scenario. The results obtained in this study, utilising mites from a largely ivermectin naïve population, represent important baseline data and will enable more detailed comparisons to be made in the future.
Chapter 8
Concluding remarks

Scabies is endemic to developing regions and indigenous populations worldwide, and is a significant disease of companion animals and livestock. In remote communities of northern and central Australia, commonly referred to as our “fourth world”, Aboriginal Australians are dying at least twenty years earlier than their non-Aboriginal counterparts (Australian Bureau of Statistics, 2004). Prevalence rates of diseases such as acute post streptococcal glomerulonephritis, acute rheumatic fever and rheumatic heart disease are among the highest in the world (McDonald et al., 2004). These conditions are increasingly being linked to streptococcal pyoderma resulting from infected scabies. The burden of scabies is particularly apparent in children, with point prevalence rates up to 65% (Carapetis et al., 1997). These rates remain high despite intensive mass treatment programs with 5% permethrin (R. Andrews, pers. comm.) In addition to the morbidity caused by high levels of ordinary scabies, crusted scabies, an extremely debilitating form of the disease, is also more common in this region.

The addition of oral ivermectin to the limited arsenal of acaricides was once anticipated to revolutionalise control of this disease, perhaps even leading to its eradication (Burkhart et al., 1997; Lawrence et al., 1994). More than 100 million doses of ivermectin have been administered in the control of onchocerciasis and other filarial diseases (Richard-Lenoble et al., 2003). Ivermectin is now increasingly used worldwide in the treatment of ordinary scabies, and has been successfully implemented into mass treatment programs for scabies in the Solomon Islands (Lawrence et al., 2005).

In northern Australia, ivermectin has been used for over ten years in the management of crusted scabies. One recurrent crusted scabies patient has received approximately 130 doses over a 12-year period, arguably the highest in the world. Considering this, we are well poised to investigate the use of this acaricide for scabies. Despite its promise, where scabies is concerned, ivermectin is far from a “wonder drug”. There
are still several unresolved issues regarding its use for both ordinary and crusted scabies, including optimal concentration, number of doses and dose intervals. Relatively little is known about the therapeutic concentrations of ivermectin in the skin, particularly in crusted scabies. A major limitation of its use is that ivermectin is contraindicated in young children, pregnancy and lactation, and these groups probably constitute the primary reservoirs and transmitters of scabies in the community. There are few randomised controlled trials concerning the safety and efficacy of ivermectin for scabies, and this needs to be addressed as a matter of priority.

This PhD project was initiated due to concerns regarding the long term efficacy of ivermectin as a treatment for crusted scabies. Since its introduction in 1995, treatment failures have repeatedly been observed with ivermectin therapy for crusted scabies, despite intensive multiple dose regimens. This culminated in 2000, with clinical and in vitro ivermectin resistance documented in two crusted scabies patients (Currie et al., 2004). Analysis of ten years of in vitro data shows that median survival times to ivermectin have doubled since its introduction (chapter 3). This raises serious concerns regarding the sustainability of this relatively new drug for scabies. In light of the increasing use of ivermectin, and its likely incorporation into mass-treatment programs, it was critical to begin to define the mechanisms of ivermectin resistance in scabies mites.

Molecular studies on *S. scabiei* have been historically limited due to difficulties in mite identification, low parasite burden (in ordinary scabies), and insufficient access to animal models. This has been vastly improved by the construction of *S. scabiei* cDNA libraries and resulting gene discovery project, which facilitated most of the work presented in this thesis. However, mite supplies for continuing projects are still limited, and are currently reliant on the sporadic admission of crusted scabies patients to Royal Darwin Hospital. A major impediment to this work has been lack of access to mites with a clear ivermectin resistance phenotype. When using mites obtained from the clinical setting, there are inevitable confounding factors such as unequal selection pressures, physiological factors and the co-administration of other drugs, making definition of resistance difficult. Clearly, if we are to make true headway into the study of acaricide resistance in *S. scabiei*, the development of an
animal model is imperative. Work towards this objective is progressing well, meaning that more clearly defined research will be possible in the future.

Even without the above impediments, it is important to acknowledge that the molecular mechanisms of ivermectin resistance are complicated. Although early studies in the nematode *H. contortus* suggested that a single dominant gene controlled resistance, it is now evident that ivermectin resistance is multi-factorial, and probably differs between even closely related species. While several molecules have been implicated in ivermectin resistance, there is still little functional evidence to support any of these, nor are there any reliable genetic markers available.

This study involved the first identification of candidate ivermectin resistance genes from *S. scabiei* var. *hominis*. Starting from a knowledge base of zero, this was a substantial achievement in itself. An *S. scabiei* EST dataset was utilized to identify members of the ABC-Transporter superfamily (chapter 4). Of particular interest were the P-glycoprotein and multi drug resistance-associated proteins, which have been implicated in ivermectin resistance, there is still little functional evidence to support any of these, nor are there any reliable genetic markers available.

Of considerable significance was the identification and characterization of SsCl- a novel pH-gated chloride channel from *S. scabiei* (chapter 5). This class of ligand gated ion channel has only recently been described (Schnizler et al., 2005), and this is the first evidence that such channels exist outside insects. Most importantly, this channel was shown to be irreversibly activated by ivermectin. It therefore may represent an important drug target and subsequent resistance candidate in the scabies mite. Presently, nothing is known about the physiological function of pH-gated chloride channels. Future drug-binding and immuno-localisation studies may shed more light on its role in *S. scabiei*. If SsCl is a drug target as predicted, the genetic diversity of this gene highlighted in chapter 7 suggests that ivermectin treatment may possibly select for a channel with altered ivermectin binding properties, facilitating the rapid development of resistance.

Quantitative reverse-transcriptase PCR (qRT-PCR) was applied to scabies mites to measure transcription levels in candidate ivermectin resistance genes (chapter 6). The major finding of this work was the up-regulation of a delta-class glutathione S-transferase and a multi drug resistance protein in ivermectin exposed mites. Although
both these protein classes are known to confer drug resistance in other organisms, this is the first molecular evidence of a possible association with ivermectin exposure. This has significant implications in the study of ivermectin resistance in other parasites.

The work in thesis forms a foundation for continued studies on ivermectin resistance in scabies mites, with the ultimate aim to develop a suitable diagnostic test for ivermectin resistance at the community level. For this to occur, future studies should incorporate the following points:-

- As described above, virtually nothing is known about the therapeutic concentrations of ivermectin at the site of infestation, especially in crusted scabies. In order to predict the pharmacodynamics of the therapeutic situation and how this relates to selection for resistance, it is imperative that ivermectin levels are determined *in situ* and compared to the sensitivity of all developmental stages of *S. scabiei*.

- Development of known resistant and susceptible lines in an animal model, enabling clearer correlations to be made between ivermectin resistance phenotype and corresponding genotypic changes.

- To develop a reliable molecular test, measurement of allele frequency should be continued, both in candidate ivermectin resistance genes, in addition to genes unrelated to drug targets to test for population bottlenecks during selection.

The deleterious impact of scabies on the health of Aboriginal Australians is without question. This study represents major advances in our understanding of the genetics of *S. scabiei*, with the characterisation of key genes potentially associated with ivermectin resistance. This will enable the development of molecular techniques to facilitate continued monitoring, and enable the identification of emerging ivermectin resistance in scabies endemic communities. This is particularly important in light of increasing pressure from health professionals to begin mass intervention programs with ivermectin in remote communities.
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