# MOLECULAR CLONING AND CHARACTERIZATION OF A VESICULAR ACETYLCHOLINE TRANSPORTER FROM *ONCHOCERCA VOLVULUS* AND ITS BIOCHEMICAL CHARACTERIZATION IN *HAEMONCHUS CONTORTUS*

**BY**

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**A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY OF MAKERERE UNIVERSITY**

**SEPTEMBER, 2009**

## DECLARATION

I, **Nanteza Ann,** declare that this thesis is my original work and it has never been submitted for a degree award in this or any other university.

Signature……..Date ……

This thesis has been submitted with the approval of the following supervisor:

Professor George William Lubega,

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Signature …….Date…….……

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## DEDICATION

To my loving parents

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| ACh | Acetylcholine |
| ANOVA | Analysis of variance |
| APOC | African programme for onchocerciasis control |
| ATP | Adenosine tri-phosphate |
| APS | Ammonium persulphate |
| BSA | Bovine serum albumin |
| bp | base pair |
| Bzs | Benzimidazoles |
| $CaCl_2$ | Calcium chloride |
| cDNA | Cloned deoxy ribonucleic acid |
| ChAT | Choline acetyltransferase |
| cm | Centimeter |
| $C_{max}$ | Concentration of drug causing maximum response |
| dATP | Deoxy adenosine triphosphate |
| dCTP | Deoxy cytosine triphosphate |
| DEC | Diethyl carbamazine |
| DFP | Di-isopropyl phosphofluoridate |
| dGTP | Deoxyguanine triphosphate |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxy ribonucleic acid |
| Ds | Double stranded |
| DTT | Dithiothreitol |
| dTTP | Deoxy thymine triphosphate |

| | |
|---|---|
| $E_{max}$ | Maximum response |
| EDTA | Ethylene diamine tetra acetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| FCS | Foetal calf serum |
| Fig | Figure |
| HCl | Hydrochloric acid |
| Ho | Null hypothesis |
| IPTG | Isopropyl-I-thio-β-D galactosidase |
| IVM | Ivermectin |
| kb | Kilo base |
| kDa | Kilo-dalton |
| KCC | Kampala City Council |
| Km | Kilometer |
| LB | Luria broth |
| M | Molar |
| MATS | Monoamine transporters |
| mRNA | Messenger ribonucleic acid |
| ml | Milliliter |
| mM | Millimolar |
| MDP | Mectizan donation program |
| Mf | Microfilaria |
| MPP | N-methyl-4-phenylpyridinium |
| MPTP | N-methyl 1,2,3,6-tetrahydropyridine |
| NaCl | Sodium chloride |

| | |
|---|---|
| NCBI | National Center for Biotechnology Institute |
| ng | nanogramme(s) |
| $N_iT$ | Number of immotile worms in the test group |
| Nm | Initial number of motile worms in the rest group |
| No. | Number |
| OCP | Onchocerciasis control programme |
| ORF | Open reading frame |
| P | Level of significance |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PEM | Pipes, ethylene diamine tetra-acetic acid, Magnesium sulphate |
| PMSF | Phenyl methyl sulfonyl flouride |
| Pfu | Plaque forming units |
| PI | Percent inhibition |
| Pmol | Picomoles |
| RACE | Rapid amplification of cDNA ends |
| RBC | Red blood cell |
| RNA | Ribonucleic acid |
| RPM | Revolutions per minute |
| SDS | Sodium dodecyl sulfate |
| SL | Sibling |
| TAE | Tris acetate ethylene diamine tetra-acetic acid |
| TEMED | N,N,N',N'-Tetramethyl ethylene diamine |
| TM | Transmembrane |

| | |
|---|---|
| UTR | Untranslated region |
| U | Units |
| UV | Ultra violet |
| VAChT | Vesicular acetylcholine transporter |
| VAT | Vesicular amine transporter |
| VMAT | Vesicular monoamine transporter |
| WHO | World health organisation |
| w/v | weight per volume |
| × g | gravitational force |
| β | Beta |
| % | Percent |
| $^{o}$C | Degrees celcius |
| μM | Micromolar |

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## ABSTRACT

*Onchocerca volvulus* is a human subcutaneous parasitic nematode recognized throughout the world as the single most common cause of irreversible blindness. No drug currently available is completely safe and effective for mass treatment against the adult worms. The identification of suitable drug targets is therefore an essential undertaking for onchocerciasis control. Cholinergic neurotransmission depends on the regulated release of acetylcholine. This requires the loading of acetylcholine into synaptic vesicles by the vesicular acetylcholine transporter (VAChT). Vesicular acetylcholine transport is essential for cholinergic neurotransmission because homozygous VAChT knockout mutants in *Caenorrhabditis elegans* and *Drosophila melanogaster* do not live for more than a few days.

In this study, the *O. volvulus* putative vesicular acetylcholine transporter (*Ov*VAChT) gene was cloned and characterized. The predicted *Ov*VAChT partial protein is composed of 404 amino acids and contains 11 conserved putative transmembrane domains. The cloned VAChT cDNA forms approximately 75% portion of the gene in comparison to *C. elegans* unc 17 gene homologue. It contains the 5' end of the gene and also has 5' untranslated region which contains 122bp from the adaptor ligated site.

The *Ov*VAChT putative protein shows extensive homology to the vesicular acetylcholine transporter, *C. elegans* unc17 gene (98%). It is also closely related to other vesicular acetylcholine transmitter transporters from *Drosophila melanogaster* (56%), *Anopheles gambiae* (50%), *Torpedo californica* and other *Torpedo species* (55%) that have been reported. Relative to the amine transporters, the *Ov*VAChT bears a closer

relationship to the rat vesicular monoamine transporters VMAT1 (37%) and VMAT2 (39%). In addition, it also displays a weak similarity to a class of bacterial drug resistance and membrane transporters (22%). Alignment of the amino acids for the VAChTs and MATs from various animal species showed high conservation within the transmembrane regions with charged amino acids indicating functional significance of the gene in substrate transportation.

Phylogenetic analysis of VAChTs and MATs clustered the Onchocerca gene in the same clade with *C. elegans* suggesting the close evolutionary relatedness of the two nematodes. The three remaining clades of the neighour-joining tree contain the other invertebrates' and vertebrates' VAChTs in two clades and all the monoamine transporters (MATs) in a single clade indicating greater conservation of MATs compared to VAChTs amongst various animals. The phylogeny tree revealed that the nematodes diverged from the ancenstry much earlier in evolution in relation to other animals. The remaining three clades have close evolutionary relationship and diverged from the ancenstry almost at the same time but much later than the nematodes.

The presence of a 70kDa protein band in rat brain and its absence in *H. contortus* or *O. volvulus* total protein extracts while blotting using rat anti-VAChT antibodies designed from the C-terminus cloned rat VAChT in Western blotting, conformed with the documented marked variations of the amino acid sequences within this region. Thus, the 3' end can be used in designing specific anti-VAChT antibodies for different animal species.

An additional helminth/nematode species which is parasitic, has now been found to contain a VAChT gene similar to those found in *C. elegans* (unc17) and other invertebrates and vertebrates which are free living organisms. Since unc17 mutations

protect against organophosphorus toxicity and the Torpedo electric lobe provides extremely dense cholinergic innervation to the electric organ, these relationships support a role of VAChT of *O. volvulus* (*Ov*VAChT), in neurotransmission. This molecular cloning and characterization of (*Ov*VAChT), the *C. elegans* unc17 gene homologue, will provide unlimited amounts of materials for further studies.

To address the potential role in disease and in drug and/or vaccine development, recombinant *Ov*VAChT protein will be prepared so that anti-sera can be raised and used to more precisely define the localization of this protein in the worm. Furthermore, it may be possible that a comparison of the inhibition characteristics of the recombinant *O. volvulus* and human VAChT proteins may reveal compounds which can specifically inhibit the parasite protein. Alternatively, non-cross-reactive antibodies may be useful in tackling the parasite's defense systems.

In the absence of a suitable animal model for screening compounds with potential macrofilaricidal activity against human onchocerciasis, the availability of viable specimens from nodule digestion for laboratory studies is extremely limited. Vesamicol, 2-(4-Phenyl piperidinol) cyclohexanol (AH5183) is a compound that blocks *in vitro* and *in vivo* acetylcholine accumulation in cholinergic vesicles. It blocks the storage of acetylcholine into synaptic vesicles by binding to the VAChT protein. Consequently, nerve transmissiom across the synapse is inhibited leading to loss of motor activity resulting into paralysis which is characterized by loss of motility of the organisms.

This study further characterized the VAChT protein biochemically in the related *Haemonchus contortus* nematode adult worms that are easier to obtain and maintain *in vitro*. The effect of vesamicol on motility of *H. contortus* female adult worms *in vitro*

was assessed, to obtain some insights before going into doing the expensive venture with use of *O. volvulus* worms. The worms were exposed to various dilutions of the vesamicol inhibitor at concentrations of 0.1mM, 0.3mM, 0.5mM, 0.7mM, 0.9mM and 1mM, in physiological saline at 37$^{o}$C and worm motility was monitored at 30 minutes intervals for 3 hours. A time-response graph plotted from three representative vesamicol concentrations (0.1mM, 0.5mM and 1mM), revealed a general increase in the mean percent inhibition of worm motility with increasing vesamicol concentration and incubation period.

Vesamicol dilutions at 1mM reached an Emax of 100% inhibition of worm motility after 2 hours of incubation, while 0.5mM and 0.1mM vesamicol concentrations never attained 100% inhibition even at the end of the 3-hour incubation period. Analysis of variance between the mean percent inhibitions of worm motility revealed a significant difference in the mean % inhibition caused by the different vesamicol concentrations ($p<0.0001$) at the different periods of incubation ($p= 0.007$), suggesting that vesamicol was effective against *H. contortus* female adult worms *in vitro*. Both the concentration of vesamicol and incubation period were found responsible for the observed increase in percent inhibitions of worm motility. The results of this study provide the first insights into the effect of VAChT inhibitor in viable organisms.

**Key words:** *Onchocerca volvulus*, Vesicular acetylcholine transporter, *Haemonchus contortus*, *In vitro*, Inhibition, Vesamicol.

# CHAPTER ONE

## INTRODUCTION

Filariasis afflicts over 100 million people in Asia, Africa and the Americas (Ottesen, 1995), with close to 400 millions being at risk of infection in third world countries (WHO, 1987). Onchocerciasis, or river blindness caused by the human parasitic nematode, *Onchocerca volvulus* is recognized throughout the world as the single most common cause of blindness, affecting approximately 360,000 people in this way, with a further 75 - 80 million people at risk of contracting the disease (Cook, 1990). It is estimated that of the 17.5 million people actually infected with *Onchocerca volvulus* (Ramachandran, 1994), 95% live in a zone across Africa spreading from 15$^{o}$N to 15$^{o}$S (including the Sub-Saharan tropical belt) presenting huge social, economical and medical problems in these areas (Weatherall, 1996). The scale of the problem and debilitating effects of the disease makes it a condition of major public health importance across the whole of Africa (Tchakoute *et al*., 1999).

The disease is characterized by pruritis and visual impairment associated with a spectrum of skin and eye lesions. Adult worms induce the formation of subcutaneous nodules, but the most clinically significant disease is brought about by the microfilariae that invade dermal or ocular tissues (Buck, 1974). Onchocerciasis is not a fatal disease, but in the absence of effective treatment, the lesions described are progressive to the point of severe disability. Untreated human onchocerciasis may result in blindness (Buck, 1974). Successful treatment could potentially improve the lives of millions of individuals by halting the progression and even reversing the symptoms.

The drugs in current use are not satisfactory because they are either not effective against all parasite stages or extremely toxic**.** The current mainstay of chemotherapy,

ivermectin kills only the immature (microfilaria) stages but not adult worms which may live in infected individuals for 15-20 years (Johnson, 1995). There is, therefore, a clear need for the development of alternative drugs which are single dose and safe macrofilaricidals. Since no alternative treatments are available for routine therapy, and in large parts of the *O. volvulus* endemic areas people are receiving similar regimes of ivermectin treatment, the appearance of ivermectin resistance in *O. volvulus* could lead to the failure of the onchocerciasis control programme and leave millions of people exposed to eventual infections.

Ivermectin resistance has been reported in the literature in gut nematodes of cattle, sheep and goats world wide (Carmichael *et al.,* 1987, Oosthuizen and Erasmus, 1993; Pornroy and Wbelan, 1993). Laboratory selected and field strains of *Oesophagostomum species* resistant to ivermectin have been isolated from pigs (Pornroy and Wbelan, 1993). Ivermectin resistant mutants of the free living nematode, *Caenorhabditis elegans* have also been selected (Johnson, 1995). Resistance to ivermectin has been reported after 12 to 32 treatments of livestock over 1 to 5 years whereby the treatment of nematodes in livestock is often much higher than that in human onchocerciasis prevention. In endemic areas, 16 - 20 treatments of people over 8 to 10 years, may apply a similar selection pressure to that which has resulted in ivermectin resistance in nematode parasites of livestock (Johnson, 1995).

No molecular target for vaccine based protection against filarial infections has been convincingly identified. Thus, improved methods for prevention and/or treatment of infections by filarial nematodes are urgently required. The established WHO-Macrofil Chemotherapy Project aims at developing such a safe macrofilaricide, ideally effective by a single dose (Renz *et al.,* 1995). It was, therefore, essential to initiate studies which

might lead to development of single dose and safer drugs which are effective against adult worms. The identification and characterization of suitable drug targets is a strongly justifiable undertaking. The vesicular acetylcholine transporter is one possible drug target which appears to be essential for survival at least in *C. elegans* (Alfonso *et al.,* 1993) and *Drosophila melanogaster* (Kitamoto *et al*., 1998).

The neurotransmitter acetylcholine (Ach) (Wu and Hersh, 1994) is synthesized in a one step reaction by choline acetyltransferase (ChAT); acetyl-CoA: choline O-acetyltransferase. In the cholinergic nerve endings, most of the ACh produced by ChAT is transported from the cytoplasm into the synaptic vesicles, which clearly plays a role in the storage of the neurotransmitter (Israel *et al*., 1970). Acetylcholine accumulation in the vesicles involves the activity of an H+-pumping ATPase, which generates an H+ electrochemical gradient (Anderson *et al.,* 1983). This gradient is utilized by a vesicular ACh transporter to drive ACh uptake via proton exchange (Parsons *et al*., 1993). A similar mechanism has been described for the transport of biogenic amines into synaptic vesicles (Johnson *et al*., 1979).

The gene, unc17, which encodes the putative vesicular transporter in the free living nematode, *Caenorhabditis elegans* has been identified (Alfonso *et al.,* 1993). Evidence for a functional activity of the vesicular acetylcholine transporter gene was shown by mutations in the gene which revealed impaired neuromuscular function that lead to jerky, coiling locomotion (Brenner, 1974; Rand and Russell, 1984) and abnormal pharyngeal pumping and defeacation (Avery and Horvitz, 1990). Since acetylcholine is the excitatory neurotransmitter at nematode neuromuscular junctions (Chalfie and White, 1988), unc17 might be involved in cholinergic processes. Brenner (1974) also observed that unc17 mutants were resistant to cholinesterase inhibitors. The direct effect of

cholinesterase inhibition is a rise in the synaptic concentrations of acetylcholine and this did not occur in unc17 mutants, possibly due to acetylcholine transport failure. Additional evidence for cholinergic involvement came from the accumulation of high concentrations of acetylcholine in certain other unc17 mutants (Hosono *et al.,* 1989) and the genetic interactions between unc17 and the closely linked ChAT gene which encodes choline acetyltransferase (the biosynthetic enzyme of acetylcholine) (Rand and Russell, 1984; Rand, 1989). In some unc17 mutants, cholinergic function is completely lost and the phenotype is lethal (Alfonso *et al*., 1993). This observation, therefore, suggests that unc17 is essential for survival.

Two rat proteins have sequence similarity to unc17: (i) the synaptic vesicle monoamine transporter (SVAT; VAT1) and (ii) the chromaffin granule amine transporter (CGAT; VAT2). These proteins transport biogenic amines (catecholamines, serotonin and perhaps histamine) into synaptic vesicles or chromaffin granules (Erickson *et al*., 1992; Liu, 1992) and are 62% identical to each other (Liu, 1992). The *C. elegans* unc17 protein is 37% identical to the rat CGAT and 39% identical to the rat SVAT, and appears to belong to the same gene family of proteins which have 12 transmembrane domains (Liu, 1992). These characteristics further suggested that unc17 encodes a vesicular neurotransmitter transporter protein.

This study postulated that if the *O. volvulus* unc17 gene homolog exists, the protein it encodes is probably vital for survival of *O. volvulus* and its inhibition might be macrofilaricidal. The basis for this is that, vesicular acetylcholine transporters (VAChTs) have been cloned and sequenced from other organisms namely, three species of Torpedo (Erickson *et al.,* 1994), rats (Roghani *et al*., 1994), humans (Varoqui *et al*., 1994), Drosophila (Kitamoto *et al*., 1998) and Anopheles (Berrard *et al*., 1998). The deduced

protein sequences present similarities to each other, *C. elegans* unc17 and to the two rat vesicular transporters of biogenic amines (SVAT and CGAT) (Erickson *et al*., 1992; Liu, 1992; Erickson and Eiden, 1993). These vesicular transporters/$H^+$ antiporters constitute a family of proteins that differ from the plasma membrane transmitter transporters (Henderson, 1993) and that share common structural features; (i) 12 transmembrane domains (TM) spanning the vesicular membrane; (ii) charged amino acid residues within transmembrane domains that may be involved in substrate transport; (iii) a glycosylated and less well conserved luminal loop located between TM1 and TM2; (iv) cytoplasmic N- and C-termini that display weaker similarities than the rest of the protein (Erickson *et al*., 1992; Liu, 1992; Alfonso *et al.,* 1993; Erickson and Eiden, 1993; Varoqui *et al.,* 1994).

Vesamicol, 2-(4-Phenyl piperidinol) cyclohexanol (AH5183) is a compound that blocks *in vitro* and *in vivo* acetylcholine accumulation in cholinergic vesicles. It blocks the storage of acetylcholine (Ach) into synaptic vesicles by binding to the vesicular acetylcholine transporter protein (VAChT) (Marshall, 1970; Parsons *et al*., 1993). Consequently, nerve transmission across the synapse is inhibited leading to loss of motor activity resulting into paralysis, which is characterized by loss of motility of the organisms. The inhibitory effects of vesamicol have been demonstrated on purified cholinergic synaptic vesicles isolated from the electric organ of the Marine Ray Torpedo (Anderson *et al*., 1983; Barh and Parsons, 1986b) and on purified rat brain synaptic vesicles (Haigh *et al.*, 1994).

*Haemonchus contortus* is a parasitic helminth belonging to the family Trichostrongyloidea. It is the type species of the genus Haemonchus and it occurs primarily in sheep and goats. Its predilection site is the abomasum of the ruminants and

its distribution is world wide, occurring mainly in the tropical and sub-tropical regions, including East Africa. *Haemonchus contortus* is one of the most pathogenic nematodes of small ruminants and it is the most prevalent nematode in small ruminants (Soulsby, 1982).

Nematode viability has gained interest in the search for techniques to use for evaluating or screening anthelmintics and/or optimization of conditions for cryopreservation. Previous attempts to quantify viability have relied primarily on observation parameters such as worm motility, larval development, growth, moulting, microfilarial release and pharyngeal pumping. Though these are time consuming and inherently inaccurate owing to the often very subjective nature of the visual assessment involved, they are cheap and affordable for preliminary work. More objective semi-automated tests, however, have also been developed and include tubulin polymerization (Howels and Delves, 1985), motility indices (Bennett and Pax, 1986) and post-incubation worm migration (Stables *et al*., 1987).

Screening for compounds with potential macrofilaricidal activity against *Onchocerca volvulus* in humans is still a problem (Chappell, 1985). Very few compounds have been very effective *in vivo* against *O. volvulus* that determination of the predictability of the assays used to detect possible filaricidal compounds cannot be done with much confidence. Suramin, the only macrofilaricidal drug clinically in onchocerciasis in man, is marginally effective at non-toxic doses in laboratory rodent models. Mel W, a patent arsenical which has shown effects against *O. volvulus* in trials in infected patients is not detected by the tertiary assays involving *Onchocerca gibsoni* in cattle (Copeman, 1979).

Faced with these difficulties, a wide range of assays have been evaluated in recent

years, including those which measure effects *in vitro* against filariae of laboratory rodents and also against other *Onchocerca species* (Courl *et al*., 1986; Nawale *et al*., 1987;; Pax *et al*., 1988). In the absence of a suitable animal model for human onchocerciasis, the availability of viable specimens from nodule digestion for laboratory studies is extremely limited. As a result, laboratories engaged in search for novel macrofilaricidal agents have to employ other filariae (models), for instance *Acanthocheilonema vitae* or *Brugia pahangi* in rodents as screening models for compounds with potential anti-Onchocerca effects (WHO, 1982; Jacquet, 1986).

In this study, vesamicol was used on *H. contortus* female adult worms to verify whether it has any effect on these nematodes, since they are easy to obtain when still fresh. Promising results obtained from this study, may warrant going into an expensive venture with use of *O. volvulus*. A limitation to the reliability of these screens is the potentially subjective assessment of worm motility.

Cholinergic neurotransmission depends on the regulated release of acetylcholine from the vesicles. This requires the loading of acetylcholine into synaptic vesicles by the vesicular acetylcholine transporter (VAChT). Vesicular acetylcholine transport is essential for cholinergic neurotransmission because homozygous VAChT knockout mutants in *Caenorrhabditis elegans* and *Drosophila melanogaster* do not live for more than a few days. The inhibitory effects of vesamicol have been demonstrated on purified cholinergic synaptic vesicles isolated from the electric organ of the Marine Ray Torpedo (Anderson *et al*., 1983; Haigh *et al.,* 1994). As yet, there was no study, which had been carried out to assess the effects of vesamicol on viable organisms, yet VAChT is essential for the survival, at least in *Caenorrhabditis elegans* adult worms (Alfonso *et al*., 1993).

The *O. volvulus* putative vesicular acetylcholine transporter gene, the (*C. elegans* unc17 homologue) was cloned and verified for its functional identity using its gene structure/sequence in this study. Further characterization of the transporter was done by assessing the effect of vesamicol on motility of *Haemonchus contortus* female adult worms *in vitro*.

Apparently, it may be possible that a comparison of the inhibition characteristics of the recombinant *O. volvulus* and human VAChT proteins may reveal a compound(s) which can specifically inhibit the parasite protein. Alternatively, non-cross reactive antibodies may be useful in tackling the parasite's defense systems. *In vitro* motor activity will be valuable in assessing inhibitors/drugs, which act on the nervous system. The intrinsic activity of vesamicol against *H. contortus* female adult worms *in vitro* demonstrated in this study, is likely to be a desirable trait in the optimization of appropriate conditions for screening vesamicol analogues for their effects. Using this technique, a wider range of chemicals will be examined for this ability faster and in smaller quantities than are needed for *in vivo* studies.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 *Onchocerca volvulus*

### 2.1.1 Historical background

Onchocercal infection was first described in Africa by Leukart (Manson, 1893). He recounted his discovery of the parasite to Manson, who in turn, published the full description in 1893, giving Leukart credit (Manson, 1893). Earlier, O'Neil (1875) had observed the microfilariae of this filarial nematode in the skin of a patient from West Africa. Clinical onchocerciasis in Latin America was not reported until 1917, when Rebels found ocular disease associated with the presence of nodules on the fore-head of a small boy. He dissected the nodule and found that it contained the adult worms. Later he described the worm, the pathology of the disease, and the epidemiology of the infection. Moreover, he suspected that the blackfly, Simulium, was the vector, which was proved by Blacklock (1927).

### 2.1.2 Epidemiology

Onchocerciasis occurs predominantly in West Africa, but endemic areas stretch across the African continent, and there are foci of infection in Central and South America, and Yemen. In Uganda the disease is mostly distributed in Western (Kabarole and Mbarara), Eastern (Jinja) and Northern (Nebbi, Arua, Moyo) regions of Uganda. People (85 million) live in areas where onchocerciasis is endemic and thus are at risk of infection (Malatt and Taylor, 1992). The estimated 18 million people infected with onchocerciasis world wide are in need of urgent treatment. Of those infected, 3 to 4 million have skin disease, and 1 to 2 millions are blind or visually impaired (Taylor,

1990). Infection begins early in childhood, and more than 80% of the population is affected in some parts of West Africa: 30% have impaired vision and 5% are blind (Gibson *et al.,* 1989). Onchocerciasis is therefore one of the leading causes of blindness in the developing world, leading to a mean reduction in life expectancy of 13 years (Nwoke *et al.,* 1992).

The patterns and prevalence of the disease are dependent on the breeding and biting habits of the black-fly transmission vector, *Simulium damnosum S.L.* and the amount of human-fly contact (Stein, 1983). The flies prefer fast flowing, turbulent streams and rivers where the water's high oxygen content allows eggs and larvae, attached to submerged supports (plants, rocks and debris), to develop (Warren *et al.,* 1990). Transmission and infection is thus generally localised to agricultural areas near to such rivers, where it is found that adult men of 20 years or more are most commonly affected, presumably a result of the cumulative exposure of working men to the biting flies over a number of years (Warren *et al.,* 1990).

In areas where *O. volvulus* is endemic, it is normal for a scale of infection amongst the population to range from light, clinically and parasitologically undetectable to heavily infected individuals who generally show the severe signs of onchocerciasis. This latter group can include up to 15% of the population (Weatherall, 1996). The pattern of socioeconomic liability that has emerged as a result of human onchocerciasis in Africa has been particularly damaging. Because of debilitation and blindness, the patient is unable to maintain any type of productive activity for long. The dissertation of many river valleys in the savannah that are agriculturally fertile has been attributed mainly to the effects of this disease (WHO, 1995).

It has been noted that the microfilariae of the savannah form of the parasite are a great deal more pathogenic that those of the forest form. Its apparently increased corneal pathogenicity might be one of the factors causing the high blindness rates in the savannah, much of which is attributable to scleritizing keratitis.

### 2.1.3 The parasite

*Onchocerca volvulus* is spread by black flies belonging to the genus Simulium, usually *S. damnosum S.L* and *S. naevei.* These blackflies breed in fast flowing rivers, the humans get bitten extensively when working along or washing in these rivers. With its meal of blood, the female blackfly ingests skin dwelling microfilariae which then go through 2 further development stages over some 6 to 8 days until they become infective larvae (L3) in the proboscis. When the blackfly bites again, the larvae of *O. volvulus* escape through the membraneous labrum into the wound and penetrate the tissues to develop into adult filaria which are found clinically in nodules scattered around the body.

Approximately, 80% of the nodules, which may vary from the size of a split pea to that of the golf ball, contain 1 or 2 male and 2 or 3 female worms (Duke, 1990), in exceptional cases, more than 50 worms may accumulate in one nodule. The females are very long and thin (20 to 80cm x 0.25 to 0.45mm), and they can live for about 9 to 14 years. During this time they produce millions of living embryos (each about 220 – 300 microns long) known as microfilariae (Schultz-key, 1990). Each day, 700 to 900 microfilariae are actively released one by one by the female worm (Schultz-key, 1990). Development to the point when the female worms begin to produce microfilariae takes about one year (10 to 16 months).

### 2.1.4 The vector complex

In all countries where onchocerciasis is endemic, the vectors which have been identified as being of most importance are the *Simulium species*. These are small biting black flies, found in swarms near free running well-aerated streams (Itall, 1977). Several hundred eggs are laid in a gelatinous mass on the under side of stones or other objects in the water. When the larvae hatch they attach to submerged objects, including river crabs, and feed on algae, protozoa or small crustacea. Six or seven moults occur, then a cocoon is spun. The mature larva emerges from this, pupates and then the young fly emerges in a gas bubble and flies away.

Work carried out at the Vector control unit for human onchocerciasis, Fort Portal, Western Uganda, has investigated transmission of Onchocerca microfilaria by simulid flies. Gravid adults are caught whilst flying around the streams, and then dissected to look for onchocerca larvae. It is reported that the most important simulid vectors are *S. damnosum* and *S. naevei* (Garms, 1992), with *S. neavei* being specific to *O. volvulus* transmission.

Several morphologically similar genetic forms (cytotypes) of the *S. damnosum* complex are known to occur in Uganda (Garms *et al.,,* 1992) and these can be distinguished by different banding patterns of their larval chromosomes. There is conflicting evidence regarding ability of these cytotypes to harbour and develop *O. ochengi* microfilaria. Studies in North Cameroon suggest that the three cytotypes found there can ingest *O. ochengi* microfilaria and develop a significant proportion of them to infective larvae (Wahl *et al*., 1997). However studies in West Africa have suggested that only some of them are anthropophilic and bite man, while others are zoophilic, feeding only on animals and possibly birds (Garms *et al*., 1992). Experiments using *O. ochengi*

and *O. volvulus* specific DNA probes have shown that few, if any, filarial species of animal origin were transmitted by *S. neavei* and *S. damnosum S.L.* in the study area (Fort Portal) (Fischer *et al.,* 1996).

Typical sites for Simulium breeding grounds are well aerated streams which are shaded by surrounding trees. The streams must be fast flowing, but vary in size from small tributaries, as are found in the town of Fort Portal, to the turbulent rapids of the Nile in Eastern Uganda, around Jinja.

### 2.1.5 The disease

It must be emphasized that many infected individuals in endemic areas are asymptomatic (Weatherall, 1996). Onchocerciasis, a clinical disease from infection by *O. volvulus* takes 3 predominant forms: eye disease, subcutaneous nodules (onchocercomata) and a pruritic, hypopigmented or hyperpigmented papular dermatitis. In addition, lymphadenopathy, mostly sclerosing, may also occur. Most of these manifestations are chronic, acute skin and eye symptoms being the exceptions. The lesions seem to be due to the dead and degenerating microfilariae, their death occurring naturally or after treatment. Indeed, when they are alive, microfilariae (as well as adult worms) appear to be unaffected by the immune response of the host (Ottesen, 1995).

Blindness is the most important effect of the disease. However, dermatitis is also very distressing for numerous patients, and may lead to serious psychological effects and social isolation (Weatherall, 1996). The dermatitis begins when dead microfilariae degenerate in the dermis. Pruritis is the most common symptom and scratching may be mild and intermittent or severe and unremitting. It may lead to excoriation and secondary infections. Pruritis may be the sole manifestation of the disease, especially in lightly infected individuals, and may be especially troublesome in expatriates.

### 2.1.6 Diagnosis

Four methods are available for diagnosis of onchocerciasis. The mostly used and easiest method of diagnosis is the detection of microfilariae in a bloodless skin biopsy (WHO, 1987). This `skin snip' can be obtained by lifting the skin with the tip of a needle and excising a disk of dermis and epidermis with a razor blade, or, preferably, by using a cornea scleral punch.

Snips are usually taken from one or more standard sites (buttocks, iliac crests, scapula or lower calf) depending on where the clinical manifestations are most severe. For instance, in Latin America, where most lesions are located above the shoulders, snip tests are customarily performed on the shoulders, whereas in Africa, lesions and diagnostic procedures are done mostly on the iliac crests and buttocks. It may be necessary to take multiple skin snips from patients with light infection (up to six are usually well tolerated). The snips, weighing up to 5mg, are then placed in distilled water or saline solution to allow microfilariae to emerge. Some authors suggest that tissue culture media may be useful, but there is no proven advantage. Most agree that examination under the microscope should be done after 30 to 60 minutes, but a 3 - to 4-hour waiting time may be useful (Greene, 1992). If nothing is found at this time, another examination performed after 24 hours of incubation may improve the sensitivity of the test. This may be particularly valuable in patients with a low density of microfilariae in the skin (< 3.5/mg of skin) as demonstrated by Taylor (1988).

Identification of microfilariae is 100% specific. However, in some forest regions of equatorial Africa, differentiation from *Mansonella streptocerca* must be done, by measurements or by staining with *M. persitans* (in Africa and South America) and *M. ozzardi* (in Central and South America).

The clinical detection of a typical nodule is good presumptive evidence of onchocerciasis. The definitive diagnosis can be made if adult worms can be identified in an excised nodule. Ultrasonography is a suitable non invasive technique in the differential diagnosis of the nodule; however, its use is limited in endemic areas.

The clinical recognition of intraocular microfilariae is diagnostic of onchocerciasis. This search must be performed with a slip lamp. A useful method consists of getting the patient to sit with head between the knees for $\geq$ 2 minutes. Normally the microfilariae are settled at the bottom of the anterior chamber, where they are not detectable with a slit lamp. When the sitting patient bends their head the microfilariae are resuspendend in the chamber, but settle again when the head is raised. The microfilariae in the process of settling to sediment can be shown for several minutes when using a slit lamp. In developed countries, this easy to perform and non invasive technique may be the first step in the diagnosis.

In the future, immunological tests may help in the diagnosis. A cloned OV-16 antigen has performed extremely well in the field testing in Africa with a high level of specificity and importantly a higher level of sensitivity than skin positivity (Greene, 1992). A "cocktail" of 3 antigens (OV-16 plus OV-7 and OV-11) has been studied (Greene, 1992). However, their definitive place is still to be characterized. Similarly, DNA probes are currently of limited help in the field (Greene, 1992).

Use of the Mazzotti test (administration of dimethyl carbamazine 50mg) is controversial in the modern literature. Most authors believe that this test, which is at least of great discomfort for most patients is often dangerous and should not be used for diagnostic purposes. However, some specialists still use it in patients strongly suspected of onchocercal infection with repeated negative skin snips and eye examination. A

modification of the test, with local application of 1 to 10% Dimethyl-carbamazine in anhydrous lanolin, is reported by some authors to provoke a typical erythema in infected patients without systemic reactions (Stingl *et al.,* 1984). Its use seems very confidential; more data concerning the sensitivity and specificity of this test are still needed.

### 2.1.7 Prevention and control

The occurrence of onchocerciasis is directly dependent on the simulium fly vector of the parasite, and thus prevention of human disease can be approached in two ways:

1. Inhibition of the development of simulium flies,
2. Decreasing man-fly contact.

The aims of treatment are also two-fold and make up the third and fourth methods of control of the disease.

#### 2.1.7.1 Inhibition of the development of simulium flies

This is perhaps the most practical approach of onchocerciasis prevention and, since 1974, the Onchocerciasis Control Programme (OCP) has been engaged in a large scale attempt to control the Savana species of the vector in this way (Cook, 1996 and Tehakoute *et al*., 1998). Methods include introduction of simulium predators, pathogens and parasites, geographical changes and application of larvicides to breeding sites. The success of the former method is still under trial, but problems have been experienced with the long flight range of the vector (up to 80km/day) which makes it difficult to attack the adult fly in one specific area (Cook, 1996). Also the environmental impact of introduction of a non-indigenous species is unknown and likely to be great. One possible time when introduction of parasites, pathogens or predators may be useful is when adult numbers have already been significantly reduced by other methods such as, larvicidal spraying.

Geographical changes having a positive impact on the reduction of simulium numbers include deforestation, which decreases the stretches of cool shaded water ways, preferred by anthrophilic *Simulium damnosum* and *S. neavei* species. Also the construction of dams and new river obstacles eliminates fast flowing water for variable distances upstream, but at the same time creates new downstream breeding sites. Realistically, neither of these extreme methods can be employed directly for vector control due to their cost and impracticality (Goldsmith *et al.,* 1989; Cook, 1996).

Larviciding of aquatic breeding sites of simulium uses the organophosphate "Temphos" (Abate) or biological insecticide (*Bacillus thuringiensis).* The OCP has come close to eliminating all simulium from hyperendemic breeding sites in West Africa and Kenya using this method, and a marked fall in the incidence of eye and skin lesions and in the worsening of pre-existing conditions is proof of this success. Application of the programme though to other regions is restricted for a number of reasons. Thorough and effective treatment requires that all breeding sites are reached, many of which are inaccessible by land and cover a wide area, and so costly aerial spraying using fixed wing aircraft or helicopters is necessary. Treatment of water-ways must be repeated regularly as the insecticides will be washed away quickly by fast flowing water, allowing flies to re-invade, including those from non-controlled areas (such as bordering countries not involved in the programme). Treatment must therefore include a huge area and continue beyond the life-span of the fly (20 years), all of which make the procedure expensive for most affected countries to complete. The long term effects of organophosphate treatment may be more severe than is currently apparent, but serious ecological impact is possible (Goldsmith *et al.,* 1989; Warren *et al*., 1990; Cook, 1996).

#### 2.1.7.2 Decreasing man-fly contact

Reducing the chances of man being bitten by flies could theoretically be achieved by placement of villages away from breeding sites, restricting river-side access during Simulid activity periods (that is morning and evening) and encouraging the use of topical insect repellants and long protective clothing (Itall, 1977). This though would also be successful if used in conjunction with breeding site and adult fly eradication, and considerations such as supply of alternative jobs and sources of drinking water would have to be made in order to have entire communities away from a river. An unrealistic proposition.

#### 2.1.7.3 Treatment

(i) Elimination of the immature stages (microfilaria) using microfilaricides, or

(ii) Elimination of the adult worms using macrofilaricides.

No drug effectively destroys both microfilaria and adult worms and thus, each method is disadvantaged by the populations remaining following treatment. Removal of microfilaria alone leaves adults to repopulate the skin with a new generation of larvae and continue the disease process. Macrofilaricides leave microfilaria with the opportunity for transmission of infective larvae to replace the adult population (Goldsmith *et al*., 1989). A combination of drugs attacking all stages of the life-cycle is therefore necessary for complete elimination of the infection, but health benefits may be gained from individual drugs, the types and mechanisms of which are summarized below.

The primary aim of treatment in the individual patient is to reduce the number of microfilariae, which represent the tissue-damaging stage of *O. volvulus*. The treatment of onchocerciasis has been radially improved by the introduction of ivermectin, an effective microfilaricidal drug causing few adverse effects.

#### 2.1.7.3.1 Microfilaricidal agents

##### 2.1.7.3.1.1 Diethylcarbamazine

Since the late 1940s orally administered diethylcarbamazine (DEC) has been the drug of choice for treatment of onchocerciasis, only recently being replaced by ivermectin (Mectizan). Diethylcarbamazine predominantly affects the neuromuscular system of the parasite, but also promotes host cell immune responses encouraging cytotoxicity mediated by immune factors (Brown *et al.,* 1983). The incredible efficiency and speed of the drug, in its destruction of microfilariae and thus the resulting wide spread inflammatory responses to dying organisms, is thought to be responsible for the numerous adverse reactions associated with DEC therapy. These include; an intense itching, rash, intense headache, joint pain and acceleration of impairment of visual capacity (the exact opposite of the hoped-for effect). This range of symptoms, known as the Mazzotti reaction frequently begins within 2 hours of drug administration and so, while use of DEC has been withdrawn as the treatment of choice, small doses are frequently used as a diagnostic aid by demonstration of these signs. Administration of low initial doses and concurrent treatment with aspirin and betamethasone have proved successful in reducing the severity of the side effects, but still the possible toxicity and impracticality for community - wide treatment, has led WHO to recommend alternative drugs (Brown *et al.,* 1983).

##### 2.1.7.3.1.2 Oral Ivermectin "Mectizan"

Ivermectin is a semi-synthetic macrocyclic lactone, used world-wide for its broad spectrum anti-parasitic activity against nematodes (Brown *et al*., 1983). It was first registered for the treatment of human onchocerciasis in 1987 under the trade name Mectizan. Shortly afterwards the manufacturers, Merck set up the Mectizan Donation

Program (MDP) which provides the drug free of charge upon request for large scale distribution programmes (Brown *et al*., 1983 and Weatherall, 1996). The recently established African Programme for Onchocerciasis Control (APOC) bases its control strategy around the use of this freely donated drug. Ivermectin is microfilaricidal (also has some effects on adults) by paralysis of both free and intrauterine larvae (Goldsmith *et al*., 1989).

Those microfilariae situated in the eye are not removed directly since the drug is unable to penetrate into the aqueous humour, but these migrate out within 4 weeks and are not frequently replaced (Weatherall, 1996). The steady flow of dying larvae is removed rapidly by the reticulo-endothelial system before they can degenerate and release toxins into the circulation, therefore avoiding the adverse reactions experienced with DEC. Other advantages over DEC include its relatively high effectiveness with a single oral dose (elimination of 80% of cutaneous microfilaria load) and the persistence of effect over a protracted period (Goldsmith, *et al*., 1989).

Mectizan is found to be the most suppressive if given every three months, but since this is often impractical, efforts should be made to ensure annual dosing. In sporadic cases found in non-endemic areas, it constitutes an excellent therapeutic tool with a good tolerance and high efficacy. In areas, endemic for onchocerciasis, Mectizan provides an easily accessible, effective treatment that avoids the progressive deterioration associated with highly infected areas.

Despite these seemingly ideal properties and availability for treatment on a community-wide scale, ivermectin is a suitable treatment for just 60% of the population. Its use is contraindicated in pregnant and breast feeding women, children less than 5 years and patients of poor health (Brown *et al*., 1983). Also, the very real prospect of

resistance development means future use of ivermectin may be hampered.

#### 2.1.7.3.2 Macrofilaricides

##### 2.1.7.3.2.1 Suramin

Suramin is a potentially toxic substance that acts by permanently sterilizing or killing adult worms. Until recently it was the only macrofilaricidal available (Goldsmith *et al*., 1989). Its suitability for wide-scale use is limited by a number of factors.

i) Administration is by slow intravenous (IV) injection only and so requires expertise and equipment, often unavailable in field sites.

ii) Weekly doses are necessary.

iii) The patient must rest on the day of drug administration.

iv) Low toxic index means side effects such as fever, mouth ulcers, diarrhoea, renal failure and even death are experienced.

v) Careful monitoring by urinalysis for casts and protein and subsequent dose modification is necessary (impractical).

vi) Abscesses may form around dead adult worms, causing secondary problems.

vii) Suramin's use is contraindicated in many patients (Goldsmith *et al.*, 1989).

The above list demonstrates that suramin's use is best suited for treatment of individuals rather than a nation wide problem.

##### 2.1.7.3.2.2 Amocarzine

Amocarzine is an oral treatment, recently developed in response to suramin's observed toxicity. It can kill up to 80% of adult worms, and also exerts some microfilaricidal activity. It is the latter that creates the Mazzotti type of reactions observed with DEC and thus makes the drug unsuitable for mass treatment.

#### 2.1.7.3.2.3 Ivermectin "Mectizan"

A higher and more efficient dose regime of ivermectin is necessary for a macrofilaricidal effect than for larval elimination. Monthly doses kill a small proportion of male adult worms, thus indirectly affecting microfilaria numbers by reducing the number of inseminations in the nodules. More than 30% of females remain alive 1 year post ivermectin treatment and so long term dosing (3 years or more) is required to exert some action on female adults (Renz e*t al.,* 1995). The use of Mectizan in this context therefore is expensive and reliant upon patient compliance, tolerance and lack of resistance development.

### 2.1.8 Ivermectin resistance

Ivermectin resistance is well recognized in veterinary practice. It has been detected in nematodes of goats and sheep (Shoop, 1993). Shoop (1993) urgues that onchocerciasis control programmes should administer ivermectin at the highest dose tolerable to as many microfilariae as possible are killed, and decrease risk of emergence of resistant forms. However, life cycle of *O. volvulus* makes survival of ivermectin resistant forms improbable in the short term. There is need to establish sensitive, easily interpretable *in vitro* methods to detect this resistance (Tagboto *et al*., 1994).

### 2.1.9 Nodulectomy

A question that has always intrigued those interested in the pathophysiology of onchocerciasis is whether palpable nodules represent the majority of active parasite in a particular individual. There is a discordancy between evidence surrounding the beneficial effects of surgical removal of the encysted adult nematodes. A small proportion of the adult population will be relieved, and so microfilaria numbers may also be marginally reduced. The degree of pathology associated with the nodule may also be limited,

especially with the removal of cranial masses (more common in Latin America) (Cook, 1996). Research suggests though that these benefits are minimal compared to the extreme pain and potential haematoma development following removal of the highly vascular nodule (Stein, 1983). Many adult *O. volvulus* are present outside the nodules and many are not palpable due to deep anatomical location or small size. Therefore, even removal of every identifiable nodule would have little beneficial effect on the overall parasite burden and evidence suggests new nodules redevelop quickly in those areas endemic to *O. volvulus* (Cook, 1996; Goldsmith *et al*., 1989).

In conclusion, no drug available is completely safe and effective for mass treatment against adult *O. volvulus* (Cross *et al*., 1998). The newly established WHO-Macrofil Chemotherapy Project aims to develop such a safe macrofilaricide, ideally effective by a single oral dose. It is with this objective in mind that most current research is based.

### 2.1.10 Transmembrane transport in neuromuscular transmission

Two distinct transmembrane transport activities participate in neurotransmission mechanisms involving "classical" small-molecule transporters such as acetylcholine, the monoamines, glutamate, and γ-aminobutyric acid (GABA). One transport mechanism is associated with the plasma membrane of the presynaptic neuron, and the other is affiliated with the membrane of the synaptic vesicle (Sudhof and Jahn, 1991; Edwards, 1992; Kelly, 1993; Schuldiner, 1994). In some neuronal networks (such as, monoamines) plasma membrane transporters are involved in terminating the actions of transporters by removing them from the synaptic cleft (Horn, 1990; Kelly, 1998). Such a mechanism is not required in cholinergic neurons because the acetylcholinesterase associated with postsynaptic targets can hydrolyse released acetylcholine (ACh) within a time period

enabling proper neuronal function (Silver, 1974). Rather, the plasma membrane transporter in cholinergic neurons is involved in transferring, by means of a high-affinity uptake mechanism, the precursor choline from the extracellular to the intracellular domain, where it can serve as a substrate for choline acetyltransferase (ChAT) in the synthesis of ACh (Rylett and Schmidt, 1993). The newly synthesized transmitter is then transported intra-vesicularly following interaction with the vesicular transporter for ACh (VAChT) (Schuldiner, 1994; Usdin *et al.,* 1995).

#### 2.1.10.1 Acetylcholine neuromuscular transmission

Nematodes also depend on acetylcholine for neuromuscular transmission of impulses. Acetylcholine (ACh) is formed in the cytoplasm of the nerve tissue by the acetylation of choline under the influence of choline acetylase with acetyl co enzyme A as the acetylating agent. Acetylcholine is transported from the cytoplasm into the synaptic vesicles probably by the vesicular acetylcholine transporter protein where it is stored in an inactive form, from where it is released when the nerve is stimulated.

Release of neurotransmitter substance is triggered by arrival of the axonal action potential at the nerve terminal (Klein, 1973; Winkler and Hortnagl, 1973). The actual mechanisms of transmitter release is not completely known; however, the imperative participation of $Ca^{2+}$ ion to link or couple the excitation of the membrane (action potential) with discharge of neurotransmitter from the nerve terminal is anticipated. The sources of this $Ca^{2+}$ are believed to be the interstatial space and/or superficial membrane binding sites at the axonal terminal. The action potential initiates an inward movement of $Ca^{2+}$ into the nerve terminal from these sites. Inward movement of $Ca^{2+}$ somehow triggers exocytotic discharge of neurotransmitter from the vesicles into the junctional cleft (Rubin, 1982). Each vesicle of acetylcholine contains approximately 10,000

molecules. After rapid migration of transmitter across the cleft, the mediator substance forms some type of bond with receptive areas on the post synaptic membrane. Cell surface receptors are specialized macromolecular structures of the cell that a neurotransmitter interacts with to elicit a response (Abranson and Molinoff, 1984). The acetylcholine receptor interaction is transient and abolished by hydrolysis of ACh by acetylcholinesterase enzyme localized in close proximity to the synaptic cleft and the diffusion of ACh away from the receptors (Rubin, 1982). The effect of free acetylcholine is prolonged if inactivation by hydrolysis is delayed. Certain drugs owe their pharmacological properties partly to their ability to effect such inhibition. Morphine, physostigmine and di-isopropylphosphorofluoridate possess the inhibitory property to some extent (Rubin, 1982).

These are the events as occurs in mammals. Events in the nematode are similar. As outlined below, targeting unique elements of nematode neurotransmission has attracted drug discovery efforts.

#### 2.1.10.1.1 Vesicular acetylcholine neurotransmission

Synaptic vesicles contain a bicarbonate-stimulated $Ca^{2+}$ or $Mg^{2+}$ ATPase which drives active uptake of [$^3$H] ACh (Parsons *et al.,* 1982). This appears to be linked to an internally acidic proton gradient generated by the ATPase (Anderson *et al.,* 1982). Active transport of [$^3$H] ACh fulfills a number of criteria for a specific carrier - mediated process. It is saturable, selective for ACh as compared to Ch, osmotically labile, and inhibited by cold and protein modification reagents. Because [$^3$H] ACh transport can be uncoupled from the ATPase, can be inhibited by mercurials without affecting the ATPase and also occurs (to a lesser extent) under passive conditions in the absence of ATP (Carpenter *et al*., 1980; Giompres and Liqman, 1980; Diebler and Morot-Gaudry, 1981;

Michaelson and Angel, 1981;), it is likely that a transporter for ACh exists which is different from the ATPase. In order to define better the molecular aspects of ACh transport, it is important to find drugs that exhibit specificity for different components of the system. The possibility that known drugs can act on vesicular ACh storage in intact preparations has not been studied extensively.

### 2.1.11 *Caenorhabditis elegans* unc17 gene

The unc17 gene of the nematode *C. elegans* has been cloned and sequenced (Alfonso *et al*., 1993). On the basis of sequence similarity to mammalian vesicular transporters of biogenic amines and localization on synaptic vesicles of cholinergic neurons in *C. elegans*, the unc17 gene likely encodes the vesicular transporter of acetylcholine (Alfonso *et al*., 1993). Mutations that eliminate all unc17 function are lethal, suggesting that the acetylcholine transporter is essential (Alfonso *et al*., 1993). Mutations in the unc17 gene of *C. elegans* were first described by Brenner (1974). These mutations resulted in impaired neuromuscular function that leads to jerky, coiling locomotion (Brenner, 1974; Rand and Russell, 1984) as well as abnormal pharyngeal pumping and defeacation (Avery and Horvitz, 1990). Since acetylcholine is the excitatory neurotransmitter at nematode neuromuscular junctions (Chalfie and White, 1988) these studies suggest that unc17 might be involved in cholinergic processes, probably as the vesicular transporter. Brenner (1974) also observed that certain unc17 mutants were resistant to cholinesterase inhibitors. The direct effect of cholinesterase inhibition is a rise in the synaptic concentrations of acetylcholine. Therefore genetic resistance to cholinesterase inhibition may result from decreased synthesis or release of the acetylcholine transmitter or from decreased response of the transmitter.

Additional evidence for cholinergic involvement came from the accumulation of high concentrations of acetylcholine in other unc17 mutants (Hosono *et al*., 1989) and the genetic interactions between unc17 and the closely linked cha-1 gene, which encodes choline acetyltransferase (ChAT, an acetylcholine synthetic enzyme) (Rand and Russell, 1984; Rand, 1989). The translation of the open reading frame from the first methionine codon (81 bp from the trans-splice acceptor site) gave a predicted protein (unc17) with 532 amino acids, a mass of 58.5 kD, and an isoelectric point of 5.29.

Two rat proteins, the synaptic vesicle monomine transporter (SVAT) and chromaffin granule amine transporter (CGAT) transport biogenic amines (catecholamines, serotonin, and perhaps histamine) into synaptic vesicles or chromaffin granules (Liu, 1992; Erickson *et al*., 1992) and are 62% identical (Liu., 1992). The *C. elegans* unc17 protein is 37% identical to CGAT and 39% identical to SVAT and appeared to belong to the same gene family of proteins with 12 transmembrane domains (Neyfakh *et al*., 1991). These characteristics suggest that unc17 is also a vesicular neurotransmitter transporter.

The present study hypothesized that if *O. volvulus* unc17 gene homologue exists, the protein it encodes is probably as vital for survival in *O. volvulus* and its inhibition might be macrofilaricidal, thus providing a new chemotherapeutic lead. The vesicular acetylcholine transporter (VAChT) have also been cloned and sequenced from three species of Torpedo (Erickson *et al.,* 1994), rats (Roghani *et al*., 1994), humans (Varoqui *et al*., 1994)., Drosophila (Kitamoto *et al*., 1998) and Anopheles (Berrard *et al*., 1998). The deduced protein sequences present similarities to each other, *C. elegans* unc17 and to the two rat vesicular transporters of biogenic amines (SVAT and CGAT) (Erickson *et al*., 1992; Liu, 1992; Erickson and Eiden, 1993). These vesicular transporters/$H^+$ antiporters

constitute a family of proteins that differ from the plasma membrane transmitter transporters (Henderson, 1993) and that share common structural features; (i) 12 transmembrane domains (TM) spanning the vesicular membrane; (ii) charged amino acid residues within transmembrane domains that may be involved in substrate transport; (iii) a glycosylated and less well conserved luminal loop located between TM1 and TM2; (iv) cytoplasmic N- and C-termini that display weaker similarities than the rest of the protein (Erickson *et al*., 1992; Liu *et al*., 1992; Alfonso *et al.,* 1993; Erickson and Eiden, 1993; Erickson *et al.,* 1994; Varoqui *et al.,* 1994) (Fig. 1).

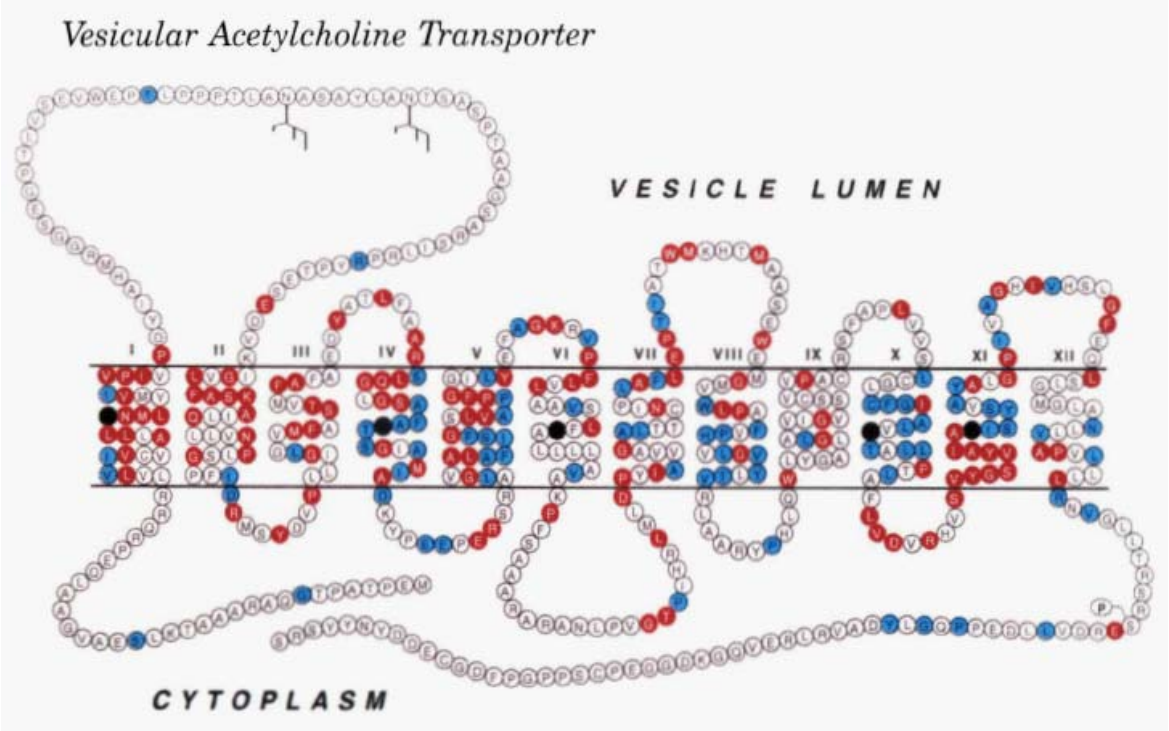



**Figure 1: Predicted rat VAChT amino acid sequence, species-conserved features of the protein, and its proposed structure within the synaptic vesicle membrane**
Twelve putative transmembrane domains (I-X11) and potential sites for N-linked glycosylation (three-pronged branches) and phosphorylation by protein kinase C (P in circle) are indicated. Red indicates amino acids conserved among all known vesicular transporters, and blue indicates amino acids unique to all VAChTs. Solid black indicates intramembrane aspartic acid residues common to all known vesicular transporters, and dark blue (in transmembrane domain N) indicates a unique intramembrane aspartic acid common in all VAChTs (Erickson *et al*., 1994).

### 2.1.12 Methods for characterization of genes

#### 2.1.12.1 Identification of cDNA clones of interest

There are three methods for screening cDNA libraries for clones of interest. They include nucleic acid hybridization, immunological detection of specific antigens and sub selection either by hybrid selection or translation of mRNA or by production of biologically active molecules.

##### 2.1.12.1.1. Nucleic acid hybridization

It is the most commonly used and reliable method for screening cDNA libraries for clones of interest. The method allows extremely large numbers of clones to be analyzed simultaneously and rapidly, does not require that the cDNA clones be full length and does not require that an antigenically or biologically active product be synthesized in the host cell (Sambrook *et al*., 1989). The method is particularly valuable in identifying small numbers of recombinant bacteriophages that carry sequences of interest in complex cDNA or genomic DNA libraries (Sim *et al*., 1979). There is a large number of different techniques that can accommodate nucleic acid probes of very different lengths and specificities. Probes utilized include homologous, partially homologous, total cDNA or synthetic oligonucleotide probes.

##### 2.1.12.1.1.1 Synthetic nucleotide probes

These are short tracts (usually < 20 bases long) of deoxy nucleotide triphosphates of defined sequence that have been synthesized *in vitro*. The sequence of these probes is deduced, using the genetic code, for short regions of the known amino acid sequence of the protein of interest (Sambrook *et al*., 1989). Because of the degeneracy of the genetic code, it is very unlikely that a given sequence of amino acids will be specified by a predictable single oligonucleotide of defined sequence. Instead, in the vast majority of

cases, the same sequence of amino acids can be specified by many different oligonucleotides. There is no way to know with certainty which of these oligonucleotides is actually used in the gene of interest. Three solutions have been found to this problem:

1. A family of oligonucleotides can be synthesized containing all possible sequences that can code for a given sequence of amino acids (Sambrook *et al*., 1989). The number of members in this family depends on the degree of degeneracy of the codons for the particular amino acids. However, if all possible oligonucleotide sequences are represented, at least one of the members will match perfectly with the cDNA clone of interest. To keep the size of each family within manageable proportions, short oligonucleotides (14-17 nucleotides) are generally used (the minimum size that is practical for hybridization). Often, more than one family of oligonucleotides is synthesized based on separate sequences of amino acids.
2. A longer (40-60 base) oligonucleotide of unique sequence can be synthesized using the most commonly used codon for each amino acid. Almost certainly, this oligonucleotide will not match exactly the sequence in the cDNA, but it will fit well enough to be detected by hybridization under non stringent conditions (Sambrook *et al*., 1989).
3. An oligonucleotide can be synthesized that contains a base such as inosine at positions of high potential degeneracy (Sambrook *et al*., 1989). Inosine can pair with all four conventional bases without seriously compromising the stability of the resulting hybrid. It is therefore possible to generate families of longer oligonucleotides that are reduced in number and yet are capable of hybridizing to virtually all cDNA clones that are likely to code for the protein of interest.

### 2.1.13 Methods to validate clones of cDNA

#### 2.1.13.1 Screening of cDNA libraries

Libraries of cDNA in appropriate bacterial cells are usually plated at high density for screening with antibody or nucleic acid probes, and any clones that react positively in the first round require several additional cycles of plating (subcloning and screening before they can be considered pure (Sambrook *et al*., 1989). However, the ability to react consistently with a particular probe, although an encouraging and necessary property is not sufficient to prove that a given cDNA clone is derived from the mRNA of interest. The only absolute proof of identity is to show that the cDNA clone contains an open reading frame that codes for the entire amino acid sequence of the protein of interest. Since this is impractical for most proteins other procedures such as PCR and Southern blotting may be more appropriate.

#### 2.1.13.2 Polymerase chain reaction

The polymerase chain reaction (PCR) allows isolation and amplification of target genes. It can be used, for example, to amplify cDNAs carried in bacteriophage vectors by using oligonucleotide primers that anneal to the flanking vector sequences (Saiki *et al*., 1988b). In some cases, this can eliminate the need for subcloning into plasmid or bacteriophage M13 vectors or for the isolation of specific fragments of DNA prior to subcloning. Because the termini of the amplified segment are defined by the sequences of the priming oligonucleotides, the polymerase chain reaction can be used to eliminate unwanted sequences flanking the target DNA. It is therefore possible to generate a series of precisely defined deletion mutants in a set of polymerase chain reactions that are primed by a progressive series of nested oligonucleotides. In addition, useful sequences (such as restriction sites) can be inserted at the termini of the amplified fragment(s) by

incorporating additional nucleotides at the 5 termini of the priming oligonucleotides. The polymerase chain reaction therefore provides a very versatile tool to increase the speed and precision of many of the methods used to manipulate DNA in molecular cloning. Amplification by PCR is used in molecular cloning and analysis of DNA including;

1. Generation of specific sequences of cloned or uncloned double-stranded DNA for use as probes.
2. Generation of libraries of cDNA from small amounts of mRNA.
3. Generation of large amounts of DNA for sequencing.
4. Analysis of mutations.
5. Chromosome crawling/walking.

#### 2.1.13.3 Southern blotting

The transfer of DNA restriction fragments from an agarose gel to a membrane is an essential technique in the analysis of genome organization and expression. This technique, known as Southern blotting, (Southern, 1975) also has important applications in the study of genetic diseases, DNA finger printing and the analysis of polymerase chain reaction products. The DNA fragments are first separated by agarose gel electrophoresis. The DNA is then denatured in the agarose gel by soaking the gel in a sodium hydroxide solution and neutralizing it in Tris buffer (Sambrook *et al*., 1989). The DNA is then blotted onto the nitrocellulose filter by capillary or vaccum action in the presence of high salt. The DNA is transferred as sharp bands which are fixed onto the filter by baking at 80$^{o}$C for at least one hour or UV translinking using a translinker machine before they are ready for hybridization.

The main drawback of this procedure is that small fragments of less than 200 base pairs in length do not transfer efficiently to nitrocellulose and may be underrepresented.

For efficient transfer of small nucleic acids, nylon membranes are recommended.

#### 2.1.13.4 DNA sequencing

The two rapid sequencing techniques in literature include the enzymatic method of Sanger *et al.,* (1977) and the chemical degradation method of Maxam and Gilbert (1977). Although very different in principle, these two methods both generate separate populations of radio labelled oligonucleotides that begin from a fixed point and terminate randomly at a fixed residue or combination of residues. Because every base in the DNA has an equal chance of being the variable terminus, each population consists of a mixture of oligonucleotides whose lengths are determined by the location of a particular base along the length of the original DNA. These populations of oligonucleotides are then resolved by electrophoresis under conditions that can discriminate between individual DNAs that differ in length by as little as one nucleotide. When the populations are loaded into adjacent lanes of a sequencing gel, the order of nucleotides along the DNA can be read directly from an autoradiographic image of the gel.

### 2.1.14 Recombinant protein expression

It refers to the directed synthesis of large amounts of desired proteins. It is often the logical next step for researchers who have identified a gene and what to study the protein it encodes. Recombinant DNA technology is used to create phages and plasmids *in vitro*, which direct the synthesis of large amounts of the products of cloned genes. A gene whose product is to be expressed is introduced into a plasmid or other vector. That vector is then introduced into living cells. Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the gene. They may also include, for example, sequences that allow their autonomous replication within the host organism, sequences that encode genetic traits that allow cells containing the vectors

to be selected, and sequences that increase the efficiency with which the mRNA is translated. Most cloning vectors utilize *E. coli* as their host organism. *Escherichia coli* have two characteristics that make it ideally suited as an expression system, for many kinds of proteins: It is easy to manipulate and it grows quickly in inexpensive media.

The basic approach used to express all foreign genes in *E. coli* begins with insertion of the gene into an expression vector, usually a plasmid. This vector generally contains several elements: (1) sequences encoding a selectable marker that ensure maintenance of the vector in the cell; (2) a controllable transcriptional promoter (such as, lac, trp, or tac) which upon induction, can produce large amounts of mRNA from the cloned gene, (3) translational control sequences, such as appropriately positioned ribosome binding site and initiator ATG; and (4) a poly linker to simplify the insertion of the gene in the correct orientation within the vector. Once constructed, the expression vector containing the gene to be expressed is introduced into an appropriate *E. coli* strain by transformation.

## 2.2 Vesamicol 2-(4-Phenyl piperidinol) cyclohexanol

### 2.2.1 Pharmacological characterization of vesamicol

The vesicular acetylcholine transporter protein is identified by the high affinity binding of vesamicol, a potent non-competitive inhibitor of acetylcholine uptake into purified cholinergic synaptic vesicles isolated from the electric organ of the Marine Ray Torpedo (Bahr and Parsons, 1986a; Anderson, *et al*., 1993). Vesamicol also acts on purified rat brain synaptic vesicles (Haigh *et al.*, 1994) and many intact and semi-intact neural preparations to block the refilling of synaptic vesicles (VP2) with acetylcholine (Bahr and Parsons, 1986a; Ricney and Whittaker, 1993). With the discovery of this marker protein (VAChT), efforts have been initiated to investigate its potential in

assessing the intergrity of cholinergic nerve terminals in neurodegenerative diseases characterized by reduced cholinergic function in humans.

Vesamicol, 2-(4-phenyl piperidinol) cyclohexanol, (AH5183) blocks transmission at cholinergic synapses through an inhibitory action on the vesicular acetylcholine uptake system. Although its principle site of action is at cholinergic synapses, it was recognized that vesamicol is also active at the sympathetic neuro-effector junction. Marshall (1970), showed that in the spontaneously contracting isolated rabbit jejunum preparations, 5μ g/ml (17μm) (+) – vesamicol produced a 75% reduction of the inhibitory effects of noradrenaline, phenylephrine and perivascular nerve stimulation but had no effect on the inhibitory effects of either isoprenaline or ATP.

**2.3 Use of motility levels to assess potency of anthelmintics**

Evaluation of motility is a useful indicator of response to anthelmintic agents, even in the case of those, which function by way of inhibition of or interference with metabolic pathways. To provide background information as to how best to evaluate potential new anti-Onchocerca compounds, a range of antiparasitic drugs at various concentrations were examined for their effects on worm motility over a seven day period (Townson *et al*., 1987). Motility was scored on a scale of 0 (completely immotile) to 10 (Normal control motility). Repeat trials showed that for the majority of compounds examined, motility levels could be used in a quantitative way to compare the relative potency of antifilarial agents *in vitro*. Further parameters of worm viability were required such as uptake and subsequent leakage of (V-14C) adenine (Comley *et al*., 1987) which might help distinguish between worms which were dead and those which were merely paralysed and possibly able to recover.

## 2.4 *Haemonchus contortus* nematode worms

### 2.4.1 General description

Haemonchus is an important genus found in the true stomach (abomasum) of sheep, goats, cattle and other ruminants (Soulsby, 1982). *Haemonchus contortus* is commonly called the stomach worm or the wire worm or the barbers' pole worm, all of which are descriptive names of this nematode. The nematodes are 10-30mm long (0.4mm diameter) and belong to the trichostrongylid group. The females are larger, more numerous than the males and have white ovaries wound spirally around the intestine which is red from the host's blood, giving – a barbers' pole appearance. Males have an even red appearance and a large bursa with barbed spicules. The eggs are 90x45 mm in size. The larvae are morphologically different at the different stages, and the diagnosis of haemonchosis in mixed infections can be based on the morphology of the third stage.

Haemonchus is among the largest genera in the superfamily, the males being 10 to 22 mm and the females 25 to 34 mm long. The worms are blood suckers and, in the freshly opened abomasum, they are easily seen, even amongst debris because of their bright red colour, and considerable size. Free of the abomasal contents, the most obvious feature of the male is the bursa, which is large enough to appear to the naked eye as a little eyelet on the end of a worm. Microscopically, the important diagnostic features are the barbed spicules and asymmetrically placed dorsal ray.The female has the white ovary and uterus twisted around the red blood filled intestine and this prominent characteristic though it is present in all the species, gave *Haemonchus contortus* its name. The female *H. contortus* has a 'tongue'-shaped vulva flap and the vulva is placed at the beginning of the posterior third of the body, a point of differentiation from Mesistocirrus, the only genus with which confusion is likely, in which the vulva is near the tip of the tail.

Close microscopic examination shows a very small buccal capsule containing a tiny lancet, which is used to pierce small blood vessels during feeding. There is a pair of easily seen, wedge-shaped cervical papillae. The fully developed adult worms feed for about twelve minutes at a time and when they move to a fresh site, bleeding continues at the former site of attachment for six to seven minutes (Soulsby, 1982).

### 2.4.2 Life cycle

*Haemonchus contortus* has a typical direct nematode life cycle (Soulsby, 1982). Embryonation starts immediately if there is oxygen, moisture and optimal temperature. At 26$^{o}$C, the first stage larva ($L_1$) is formed in 20-24 hours and development through the second stage ($L_2$) to the third stage ($L_3$) takes 4-6 days. There is no development below 9$^{o}$C. Moulting and exsheathment accompany development of larvae from one stage to the next. However, the cuticle of the $L_2$ is retained by the $L_3$, which does not feed.

The infective stage is the ensheathed $L_3$ and may live on pasture up to 6 months. Infection is by ingestion of the $L_3$, which will exsheath in rumen of sheep and migrates down the gut to penetrate the abomasal mucosa and start feeding as the $L_4$. The $L_4$ may become hypobiotic before emerging into the lumen of the abomasum to moult to the adult stage and start laying eggs after mating. The time from infection to the first appearance of eggs in feaces of the host (prepatent period) is 15-20 days. *Haemonchus contortus* has a high biotic potential whereby 5000 eggs/day/female worm may be passed in feaces (Levine, 1978).

### 2.4.3 Pathology

Anaemia is the basic feature of the infection because the worms feed on blood (Soulsby, 1982). When large numbers of larvae infect sheep, deaths can occur suddenly while the sheep still appear to be in good health. Thus as termed “acute prepatent

disease" because at this time, eggs are not seen on faecal examination. Chronic infections involving smaller numbers of worms may produce oedema (bottle jaw), iron deficiency anaemia, progressive weakness, and wool breaks and sheep death. Diarrhoea or constipation may also occur. Both fourth-stage larvae and adult worms puncture blood vessels in the stomach wall and feed on the blood that is released. Large quantities of blood spill over during the feeding process.

### 2.4.4 Diagnosis

A definite diagnosis of haemonchosis can only be made at autopsy of selected cases or by culturing of feaces and the identification of infective larvae. However, the clinical signs also may lead to a suspicion of haemonchosis. In acute cases, that is, when lambs or young sheep, which are the most susceptible, acquire a sudden severe infection, anaemia develops rapidly and the animals die without showing much more than the signs of anaemia and hydraemia.

In the more chronic cases, anaemia is also the main sign and oedematous swelling (called bottle jaw or watery poke) is frequently seen under the jaw and others may develop along the ventral aspect of the abdomen. The animals become progressively weak and later walk with a swaying gait. The skin becomes pale, and in sheep, the wool falls out in patches.

### 2.4.5 Control

The traditional approach to suppress the effects of parasitic gastroenteritis on animal production has been the use of frequent treatment, the timing of which has been largely guided by convenience and economic considerations, rather than epidemiological principles (Brunsdon, 1980). This approach led to considerable inefficiency. Resistant nematodes have, therefore, been selected (Waller, 1985). This probably explains why;

drug resistance is mostly confined to the economically important strongylid nematodes (Kelly and Hall, 1979).

The most widely used method to control parasitic worms is treatment with anthelmintics (Martin, 1985). Benzimidazoles (BZs) are widely used anthelmintics for man (Cook, 1990) and animals (Campbell, 1990) and are effective against nematodes and some trematodes. Benzimidazoles are also used as antifungal, antitumor or anti-mitotic agents (Dustin, 1984). The frequent use of anthelmintics has caused the selection of worms resistant to benzimidazoles and other anthelmintic drugs in animals (Donald, 1983). Benzimidazole resistance is a major problem for the control of *H. contortus* of sheep and other nematodes of sheep and horses (Waller and Prichard, 1986).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Cloning and characterization of *O. volvulus* VAChT gene

### 3.1.1 *Onchocerca* cDNA and genomic DNA libraries

Two primary cDNA libraries of *Onchocerca volvulus*; OVML3 (larval stage 3) ($L_3$) cDNA and Lambda Zap OV (microfilaria) (MF) cDNA libraries were obtained on dry ice from Roger Prichard, McGill University, Canada. In addition, six (6) *O. volvulus* cDNAs of different developmental stages (microfilaria, $L_2$, $L_3$, adult male and adult female), one (1) *O. ochengi* ($L_3$) cDNA and one (1) *O. volvulus* genomic library were received on dry ice from Susan Haynes, Smith College, Steven William's Lab, Nothampton, USA. All the DNA libraries were received when frozen in 7% Dimethyl sulfoxide (DMSO). All the cDNA libraries had been cloned in Lambda Zap bacteriophage cloning vector at EcoR1 (5' end) and Xho1 (3' end) restriction sites. The *O. volvulus* genomic library had been cloned into Lambda FIX 11 (Xho 1 Cut-Stratagene) vector at Xho 1 restriction site. Upon receipt, the libraries were kept at $-80^0$C until use.

Before use, the libraries were removed from the freezer and quickly thawed in the hands. Immediately, an aliquot (100µl) of each library, was diluted 1:10 using SM buffer (10mM Tris pH 7.5, 10mM $MgSO_4$, 10Mm NaCl) (900µl) and 3µl of chloroform added (0.3% final concentration). The diluted library aliquotes were stored at $4^0$C as recommended by the supplier in the shipping documents. The remainder of the undiluted libraries were kept at $-80^0$C for future use.

#### 3.1.1.1 Titering of DNA libraries

This was done to determine the concentration/titer of the diluted phage. This could be used to determine the amount of phage template in the PCR and screening

analyses. Plating of the libraries was done using Luria broth containing 10mg/ml of Magnesium sulphate (LB/$MgSO_4$) plates which had been prewarmed at room temperature for at least 30 minutes. Plating bacteria (*E. coli* XL1 Blue) (200µl), top agarose (0.7% agarose in LB medium) (3ml) and diluted phage library (1:100, 1:1000, 1:10,000) were used for initial plating in order to titer the library concentrations which were $1x10^7$ pfu/ml for OVML3 cDNA library and $1x10^4$ pfu/ml for Lambda Zap OV cDNA library after the titering. Phages were not obtained upon plating of the other libraries obtained from Smith college. The libraries where plaques were obtained upon titering, were used in the library screening by plaque lifting, however, all the available *Onchocerca* species cDNA libraries were used for PCR.

### 3.1.2 *Onchocerca volvulus, O. ochengi* and *H. contortus* worms

#### 3.1.2.1 *Onchocerca volvulus* adult worms

Human onchocerciasis patients with subcutaneous palpable *Onchocerca volvulus* nodules were identified from the Western part of Uganda (Kabarole district). Ethical clearance to use the patients was obtained from the Ministry of Health, Uganda. The nodules were surgically removed by experienced medical personnel after informed oral consent of the patients. The nodules were put individually into 50ml tubes containing RPMI 1640 culture medium supplemented with 10% fetal calf serum and 200µg/ml streptomycin. The tubes were labeled appropriately with the name, age and sex of the patient; date of collection, region/site of collection on the body, name of the surgeon and place of collection (geographical location). The tubes containing the nodules were kept on ice before delivery to the laboratory.

#### 3.1.2.2 *Onchocerca ochengi* worms

Due to complications of obtaining *Onchocerca volvulus* from humans, a survey was

made to find out if *Onchocerca ochengi* can be used to substitute or complement for *O. volvulus*. Three abattoirs (Kampala City Council, Maganjo and Fort Portal) where cattle from onchocerciasis endemic areas are slaughtered were visited. This was an attempt to obtain *O. ochengi* for use in the standardization/optimization of test procedures for *O. volvulus*.

Fresh umbilical cord biopsies (10cm diameter) were excised from the skins of slaughtered cattle. The skins were also palpated for the detection of *Onchocerca ochengi* nodules. The nodules were detected from the scrotal and udder skins. The microfilariae and adult worms were recovered from the skin biopsies and nodules respectively as described below (3.1.2.4 and 3.1.2.6).

#### 3.1.2.3 *Haemonchus contortus* adult worms

*Haemonchus contortus* adult worms were isolated from the abomasums of goats slaughtered at Kampala City abattoir. This was done for obtaining worms which were used for optimization of the DNA and RNA extractions, and other test procedures before the scarce worm material of onchocerca was processed.

#### 3.1.2.4 Screening of skin biopsies for *O. ochengi* microfilariae

Each skin sample from cattle was shaved and a small portion of each sample(2cm diameter) was cut off and put in a microtiter well containing 1ml of normal saline (0.9% NaCl) prewarmed at 37$^{o}$C for 15-30 minutes and examined under an inverted microscope. Microfilariae (MF) (1-200) were observed in some samples but were too few to warrant further work with them as they could not be purified through the sephadex column as defined in the available protocols.

#### 3.1.2.5 Isolation of *H. contortus* adult worms

The goat abomasums was opened up longitudinally and adult worms were isolated

from the abomasal wall and its contents using blunt ended forceps. The worms were washed in sterile prewarmed normal saline (0.9% NaCl) at 37$^{o}$C, after which they were put in sterile cryogenic vials and snap frozen in liquid nitrogen.

**3.1.2.6 Isolation of *O. volvulus* and *O. ochengi* adult worms**

The methodology used was adapted from that of Schultz Key *et al.,* (1977) with modifications for optimum conditions. The nodules were removed from the ice or 4$^{o}$C and transferred to fresh RPMI 1640 culture medium supplemented with 10% fetal calf serum (FCS) and 200µg/ml of streptomycin and maintained at room temperature for 15 – 30 minutes. In a sterile hood the nodules were excised of all adherent tissue using sterile equipment. A collagenase solution in water (0.3 mg/ml) containing 0.1M $CaCl_2$ was prepared and sterilized by filtering through a 0.45µm filter with the aid of a syringe. The nodules were individually put into the sterile collagenase solution. The tubes were firmly closed to ensure no leakage, placed horizontally into 37$^{o}$C incubator and allowed to rock at 150 – 200 rpm for 9 – 10 hours after which the collagenase solution was replaced with fresh solution and incubations lasted for 24 to 48 hours depending on the size of the nodules.

The enzymatic digestion was observed under sterile conditions in the hood, and worms emerging from digested nodules were carefully teased out with blunt ended forceps (to avoid tearing them) and washed with sterile RPMI 1640 culture medium. Incompletely digested nodules were put back into less concentrated and fresh sterile collagenase solution and digestion continued with observations to avoid digesting the emerging worms themselves. The isolated adult worms were inspected under an inverted microscope to ensure their intergrity. They were then put into cryogenic vials and snap frozen in liquid nitrogen prior to storage at -80 $^{o}$C.

### 3.1.3 Extraction of genomic DNA from adult worms

Liquid nitrogen was poured into a sterile frozen mortar and the frozen sample (200mg) was added. The worms were ground with pestle and mortar to a powder (mortar was kept on ice). Using a sterile frozen spoon the powder was distributed onto the surface of the lysis buffer (TENS 9 buffer = 500mM Tris-Cl at pH 9, 100mM EDTA pH 8.0, 200mM NaCl, 100μg/ml of DNase-free RNase) (5ml) per 200mg of tissue. The solution was transferred into a sterile 50ml plastic tube and shaken to obtain a homogenous solution. The tube was placed on a rocking platform (150-200 rpm) at room temperature for 10 minutes. To the tube was added 0.5ml of 20% SDS to make a final working concentration of 3% SDS. The tube was gently inverted 3-5 times and continued with rocking for 10 minutes. Proteinase K solution (10mg/ml) (0.5ml) was added to a final concentration of 200μg/ml and the tube was gently inverted 3-5 times.

The sample was incubated at 37$^{o}$C overnight on a rocking platform (150-200rpm) until the solution was clear. One volume of phenol (saturated with Tris-EDTA pH 8.0) was added. The tube was placed on a rotary shaker at low speed for 30 minutes at room temperature. The tube was spun for 10 minutes, at 400 xg, at room temperature to separate the two phases. With a wide bore pipette (0.3cm orifice), the top viscous aqueous solution phase was removed and put into a clean 50ml tube. Purification with phenol was repeated once. Then one volume of chloroform:isoamyl alcohol (24:1) was added, placed on rotary shaker for 15 minutes and then spun down as above. The top aqueous phase was removed and put into a sterile 50ml falcon tube. Care was taken not to transfer the interphase layer. Sodium acetate (pH 5.3) (3M) (0.1 volume) and two volumes of ice cold ethanol (100%) were added. The tube was inverted gently for 10-15 minutes.

The DNA was observed as stringy white material which came out of the solution. The tube was spun for 10 minutes at 600 xg and the supernatant discarded. The DNA pellet was washed with ethanol (70%), air dried and 100-200µl of TE (Tris-EDTA pH 8) were added and stored at 4$^{o}$C overnight to dissolve and consequently aliquoted and stored at -20$^{o}$C.

### 3.1.4 *Caenorharbditis elegans* genomic DNA

This was obtained from Drs. G. Warwick, School of Biological Sciences, The Flinders University of South Australia, and M. Chalfie, Department of Biological Science, Columbia University, New York, 100 USA. The DNA was used as the positive control in the subsequent procedures.

### 3.1.5 Evaluation of the quality of the DNA

The genomic DNA (*H. contortus*, *O. volvulus*, *O. ochengi* and *C. elegans*) were digested with restriction enzyme (EcoR1) (Amersham). The genomic DNA (1.0µg in 5µl) was diluted with distilled deionised water (12µl) and 10x restriction enzyme buffer (React 3) (2µl) was added. The DNA mixture was gently stirred using the 10µl pipette tip. The appropriate restriction enzyme (EcoR1) (1µl) (20U) was added and incubations were carried out at 37$^{o}$C in a water bath for 3 hours. The digestion was stopped by addition of 2µl of 0.2M EDTA, pH 8 to make a final dilution of 2µM EDTA.

After incubation, 8µl of the digested and 2µl of the undigested DNA samples were individually mixed with 2µl of 6X gel loading buffer (0.25% Bromophenol blue; 0.25% Xylene cyanol FF; 30% glycerol) and 10µl loaded into the wells on 0.8% agarose gel containing ethidium bromide (0.5µg/ml). Electrophoresis at constant voltage (100 volts) for 45 minutes was done using Tris-acetate EDTA (TAE) as electrophoretic buffer.

Following electrophoresis, the gel was viewed under UV light. The gel was photographed using a polaroid camera for permanent record keeping.

There was DNA smearing throughout the lanes in the digested samples. This indicated that the DNA was fully digested and thus of high purity. Undigested samples showed DNA bands of high molecular weight (greater than 13,000 kb) in the lanes (data not shown).

### 3.1.6 Application and difficulties in DNA extraction

The adult worms should be isolated when alive (fresh) and snap frozen in liquid nitrogen to minimize DNA/RNA degradation. However, this was always not possible for onchocerca worms because the nodules had to be digested with collagenase for long periods (12 – 48 hours or more) depending on the size of the nodule which might have lead to DNA/RNA degradation. The onchocerca nodules had to be transported on ice for more than 24 hours before reaching the laboratory for digestion which may have lead to eventual death of the worms and thus DNA/RNA degradation.

### 3.1.7 Colony amplification of *C. elegans* unc17 cDNA Clone

The *C. elegans* unc17 cDNA was obtained as a glycerol stock of transformed bacteria from J. Rand, Oklahoma Medical Research Foundation. The stock had been transformed with the pBluescript ($KS^+/SK^+$) containing *C. elegans* unc17 gene insert at the EcoR1 restriction/ cloning site. The amount received was very little, so it was necessary to culture the bacteria to obtain large amounts of the plasmid construct.

Luria broth/ampicillin (100μg/ml) plates were streaked with *C. elegans* unc17 clone bacterial material from the glycerol stock using a sterile inoculation loop. Plates were incubated at $37^oC$ overnight. Using a sterile inoculation loop, one colony was obtained from the overnight growth and inoculated into 2ml of LB/ampicillin (100μg/ml) liquid

medium. Incubation at 37°C overnight while shaking at 300 rpm was carried out. Next day, plasmid DNA was prepared using the alkaline lysis method (Sambrook *et al*., 1989) as described below.

A mini culture of 1.5 ml was aliquoted into a sterile 1.5ml microcentrifuge tube, spun at 10,000 xg for 3-5 minutes. The remaining culture (0.5ml) was kept at 4°C. After centrifugation, the supernatant was discarded leaving the pellet behind. The bacterial pellet was resuspended into 100µl of ice cold solution I (50mM glucose, 10mM EDTA, 25mM Tris Cl pH 8.0), and mixed thoroughly by vortexing and left to stand on ice for 5 minutes. In case of midi-cultures (10ml), lysozyme (20mg/ml) 10µl was added before the addition of solution II to the tube.

Freshly prepared solution II (0.2mM NaOH, 2% SDS) (200µl) was added to the tube. The tube was closed tightly and contents mixed gently by inverting the tube for 3-5 times. The tube was kept on ice for 5 minutes. Ice cold solution III (5M KOAC-60ml, glacial acetic acid 11.5ml and water to 100ml) was prepared and 200µl added to the sample. The tube was closed tightly and the contents vortexed at low speed by inverted position for 10 seconds and then stored on ice for 5 minutes. The tube was spun at 10,000 xg for 5 minutes in a microcentrifuge and the clear supernatant transferred to a fresh tube.

The DNA was precipitated by topping up the supernatant with 2 volumes of ice cold absolute ethanol. The solution was mixed vigorously by vortexing and tube kept at room temperature for 1-5 minutes. The tube was spun at 10,000 xg for 5 minutes and the supernatant discarded. The pellet was washed with 1 ml of 70% ice cold ethanol and air dried for 10 – 15 minutes. The pellet was resuspended into 50µl of TE buffer (pH 8).

The plasmid DNA (5µl) was detected under UV light on 1% agarose gel after electrophoresis and staining with Ethidium bromide (0.5µg/ml). Digestion of plasmid DNA obtained from above was done with EcoRI restriction enzyme in the appropriate buffer at 37$^{o}$C for 3-4 hours.

After digestion the reaction was stopped by adding 0.2M EDTA pH 8 to a final concentration of 2µM. The samples were electrophoresed on 1% agarose gel at 100 volts for 45 minutes. Three (3) DNA bands were observed (2.9kb for the vector, pBluescript, 1.6kb for *C. elegans* unc17 insert (coding region) and 0.45kb for *C. elegans* unc17 (non coding/ untranslated region). More plasmid DNA was digested using the same procedure. The band (1.6kb) (*C. elegans* unc17) was excised from the gel and purified as described below (3.8).

The remaining plasmid DNA containing the *C. elegans* unc17 gene insert was kept at –20$^{o}$C and the remaining 0.5ml of the overnight bacterial culture was inoculated into 50ml LB/ampicillin (100µg/ml) and grown overnight as above and used for midi-plasmid DNA extraction and bacterial glycerol stock preparation.

### 3.1.8 Purification of DNA from agarose gel

The DNA was purified using the Sephaglas Band Preparation Purification Kit (Amersham) according to the manufacturers' instructions. Briefly, the appropriate band (1.6kb) was cut out of the gel with sterile scalpel blade. The gel slice was cut into several smaller pieces, and transferred into the weighed 1.5 ml eppendorf microfuge tube. The weight of the agarose slice was obtained. If the slice weighed less than 250mg, the proceeding steps were followed as described below. If the slice weighed more than 250mg the reagent volumes were increased proportionately. Gel solubilizer or modified gel solubilizer (250µl) or for scale up (1µl for each mg of agarose) was added to the

agarose slice, vortexed vigorously and incubated at 60$^{o}$C for 5-10 minutes or until the agarose slice was dissolved. The container of sephaglas BP was vortexed to form a uniform suspension. The suspension (5µl) (for scale up 5µl for each estimated µg of DNA) was added to the dissolved gel slice, and vortexed gently.

The mixture was incubated for 5 minutes at room temperature, vortexing gently every minute to resuspend the sephaglas. The mixture was spun at 10,000xg for 30 seconds in a microcentrifuge and the supernatant carefully removed, taking care not to disturb the sephaglas pellet. Wash buffer containing ethanol (supplied in the kit) (80µl) was added to the sephaglas pellet (for scale up, 16X the volume of sephaglas added above was used). The pellet was pipetted up and down several times for resuspension. The mixture was centrifuged for 30 seconds and the supernatant carefully removed, taking care not to disturb the pellet. This step was repeated twice for a total of three washes. The tube was tapped or vortexed to partially disperse the sephaglas pellet. The tube was inverted and placed on a paper towel on the bench top. The sephaglas pellet was allowed to air dry for at least 10 minutes at room temperature.

Elution buffer (Tris EDTA pH 8) (supplied in the kit) (20µl) was added to the pellet (for scale up, (4x the volume of sephadex added above). The tube was vortexed gently to resuspend the sephaglas pellet, and incubated at room temperature with periodic agitation for 5 minutes. The sample was spun at high speed (10,000 xg) for 1 minute. The supernatant was carefully removed and placed into a clean microcentrifuge tube, while taking care not to disturb the pellet. The final yield of DNA was estimated by agarose gel electrophoresis by comparing the fluorescence of Ethidium bromide stained DNA sample with that of a standard marker DNA, under UV light. The DNA was stored at –20$^{o}$C.

### 3.1.9 PCR Amplification of cDNA or genomic DNA of *O. volvulus*

#### 3.1.9.1 Primer Design

Alignment of the amino acid sequences of known putative vesicular acetylcholine neurotransmitter transporters from *C. elegans* ("unc17") and rat (rVAChT) and two rat vesicular monoamine transporters (VMATS)(rVMAT1 and rVMAT2) revealed five regions that are strongly conserved among species (Alfonso *et al*., 1993). The amino acid stretches LLLDNML, LFASKA, LAFLE, CFGIALI and SVYAIAD were used for designing specific or degenerate oligonucleotides which would be likely to bind to the *O. volvulus* putative vesicular acetylcholine transporter gene.

When an amino acid was not absolutely conserved or when various codons had been used to encode the same amino acid, preference was given to the *C. elegans* nucleotide which is A-T rich like *O. volvulus*. The designed primer sequences were synthesized by the MidLand Certified Reagent Company, UK. Various oligonucleotide pairs were used for the PCR since the VAChT gene structure in *O. volvulus* was not known and an intron could be present between the nucleotide binding sites.

#### 3.1.9.2 PCR Amplification of *O. volvulus* DNA

Genomic DNA or cDNAs (50-100ng) were used as the template in the PCR reactions. PCR amplification was carried out in 20mM dNTPS, 1.5mM $MgCl_2$, 0.3µm of each primer and 2.5 Units of Taq polymerase (Amersham) in a 50µl reaction volume. The amplification was done using Perkin Elmer 2400 PCR machine (Perkin Elmer). Amplifications were done using varying cycling conditions dependent on the melting temperatures of the primers and variable Magnesium chloride or DNA concentrations. Positive amplification was obtained by using the following PCR conditions; initial amplification cycle for 5 minutes at 94$^{o}$C, 2 minutes at 65$^{o}$C and 3 minutes at 72$^{o}$C, and

then 30 cycles of 1 minute at 94$^{o}$C, 2 minutes at 62$^{o}$C and 3 minutes at 72$^{o}$C followed by final extension at 72$^{o}$C for 10 minutes and the samples were finally maintained at 4$^{o}$C.

Primer sets P4 (CTCGCCTCCCTCGAG) or PI (CTACTACTCGACAATATGCTC) sense and P8 (GTCAGCGATCGCGTAGACGGA) (antisense) corresponding to conserved amino acid sequences LAFLE or LLLDNML and SVYAIAD respectively amplified 366bp and 1100bp DNA products from the *Onchocerca volvulus* λzap and OVML3 cDNA libraries and adult worm genomic DNA. The PCR products were of the expected molecular weight sizes corresponding to the number of nucleotides/bases between the sequences of the VAChT's gene conserved regions of primer design. .

### 3.1.9.3 Visualization of PCR products

An aliquot of PCR products (10µl) was mixed with 2µl of 6 x gel loading buffer (6 x loading buffer: 0.25% w/v bromophenol blue; 0.25 w/v xylene cyanol, 30% glycerol in water) and loaded into the wells created by the removal of the comb on 1% agarose gel containing 0.5µg/ml ethidium bromide. A molecular weight marker (Φx 174 Hae 111 digest- Sigma), 50ng was mixed with the 6 x gel loading buffer and loaded in the adjacent well of the gel. Electrophoresis was carried out at 100 volts for 45 minutes in TAE (Tris acetate 40mM, EDTA 1mM pH 8) electrophoretic buffer. DNA bands were observed when the gel was placed over a UV light source and the gel was photographed to obtain a permanent record.

### 3.1.9.4 Recovery of PCR products from agarose gel

After identifying the amplified PCR products as above, the remainder of the PCR products with the DNA bands were loaded and electrophoresed onto the 1% agarose gel at 100V for 45 minutes. The DNA bands were sliced out of the agarose gel using a sterile scalpel blade under the UV transilluminator. After slicing out the desired bands

the rest of the gel was viewed under UV light to ensure total DNA recovery from the gel before its disposal. The DNA was purified from agarose gel using Sephaglas Band Preparation Purification Kit (Amersham) following the manufacturer's instructions as described above (3.1.8).

**3.1.9.5 Analysis of PCR products**

**3.1.9.5.1 Labelling of 1.6kb *C.elegans* unc17**

This was done using the random prime labeling kit (Amersham) according to the manufacturer's instructions. Template DNA (5ng/µl) (5ul) was added to 26.5µl of distilled deionised water and heated at 100$^{o}$C for 2 minutes to denature the DNA and then immediately chilled on ice for 5 minutes before the addition of other solutions. To the tube containing DNA was added 5µl of 10x labelling mixture, 7.5µl of dNTPS (dATP, dGTP. DTTP) each, 2.5µl Klenow segment (enzyme) 5U/µl (1µl) and 50 Uci [$\alpha^{32}$P] dCTP, 3000 Ci/mmol aqueous making a total volume of 50µl. The mixture was incubated at 37$^{o}$C for 30 minutes and 5µl of 0.2M EDTA pH 8.0 were added to stop the labelling reaction.

The unincorporated $^{32}$P and nucleotides were removed using the Qiaquick spin purification columns using PN and PE buffers supplied in the kit (Qiagen) as described below. Buffer PN (500µl) was added to the labeled mixture, pipetted up and down and then added to one Qiaquick purification column which had been placed into the collection tube. The column was centrifuged at 10,000 xg (high speed) for 1 minute in a microcentrifuge. The collection tube containing the filtrate was discarded in the radioactive waste. The column was placed into a new collection tube and 500µl of PE buffer added to the column. The column was again centrifuged at 13,000 xg for 1

minute, then the column placed into a new collection tube. Again 500µl of PE buffer was added to the column, spun and collection tube discarded. The column was put into a new collection tube and spun when empty. The collection tube was discarded. Column was placed into a new collection tube and 200µl of TE buffer (pH 8.0) was added to the column, spun at 13,000xg, then the filtrate was kept on ice. The column was placed into a new collection tube, again 200µ l of TE buffer added, spun again, the filtrate was pooled together with the first pool of TE collection. The labeled DNA/probe was kept at –20$^{o}$C or boiled at 100$^{o}$ C for 5 minutes to denature the DNA, then immediately chilled on ice for 2 minutes before use as a probe.

**3.1.9.5.2 DNA hybridization**

The purified PCR products (50ng/5µl) were loaded and electrophoresed on 0.8% Ethidium bromide stained agarose gel in 1xTAE buffer at 80 volts for 45-60 minutes. A molecular weight marker (1kb ladder) (50ng) and purified 1.6kb *C. elegans* unc17 DNA (50ng) were run in adjacent lanes of the same gel. After electrophoresis the gel was viewed briefly under UV light to avoid DNA nicking.

**3.1.9.5.2.1 Transfer of DNA to filters**

After electrophoresis and photography, Whatman 3MM filter paper and transfer membrane (Hybond N+) (Amersham) were cut to the size of the gel and wetted with distilled deionised water. The DNA was transferred from the agarose gel to the membranes using the Milli Blot-V-Transfer System according to the manufacturer's instructions (Millipore).

Briefly, the wetted filter paper and membrane were placed on the porous divider plate of the Milli Blot-V-Transfer System. A window was cut out in the center of the rubber gel gasket. The window was cut when slightly smaller than the gel (at least one half

centimeter around the edge). The rubber gel gasket (with the window cut out in the center) was laid on top of the membrane. The rubber gel gasket window was covered by the gel. Then the gel stack was fully assembled. The buffer reservoir lid was placed over the gel, the four hand wheel knobs were installed and tightened until they touched the lid. Once assembled, the transfer buffer (10xSSC) (1xSSC is 0.15M NaCl plus 0.015M $Na_2$ citrate) was pipetted onto the gel to keep the surface of the gel wet. A vacuum was then applied (constant low vacuum) and maintained for a proper transfer.

Continuous pipetting of transfer buffer onto the gel surface to maintain a steady flow through the gel was done. The transfer lasted for 45-60 minutes. After which the vacuum was released, the buffer reservoir lid removed and the positions of the gel slots marked on the membrane with a soft lead pencil. The gel was peeled from the filter, stained with Ethidium bromide (0.5μg/ml in water) for 45 minutes with gentle agitation and observed under UV light to ensure complete transfer of DNA and then gel discarded appropriately. The filter was soaked in 2xSSC for 5 minutes with gentle agitation to remove any pieces of agarose sticking on the filter. The filter was removed from the wash buffer and placed on Whatman filter paper briefly to drain away excess fluid. The DNA was fixed onto the membranes by UV translinking using a stratalinker as described by the manufacturer.

**3.1.9.5.2.2 Processing of filters**

The membranes were incubated in a prehybridization buffer (5xSSPE, 5% Denhardt's solution, 0.5% (w/v) SDS, 100μg/ml denatured salmon sperm DNA) at 65$^{o}$C for at least 1 hour, while shaking in the hybridization oven. The prehybridization buffer was drained from the membrane and replaced by the prewarmed, hybridization buffer at 65$^{o}$C containing the denatured $^{32}$P labeled 1.6kb *C. elegans* unc17 fragment, (1x10$^{6}$ cpm/ml). Incubation at 65$^{o}$C with shaking was done overnight.

Next day the hybridization buffer/probe was drained into a clean 50ml tube and kept at –20$^{o}$C for further use. Membranes were washed in 2xSSC, 0.1% SDS buffer at the hybridization temperature (65$^{o}$C) for 15 minutes and repeated. The solution was replaced with 1xSSC, 0.1% SDS buffer and washed at 65$^{o}$C for 15 minutes. Finally the membranes were removed from the wash buffer, placed on blotting paper to remove the excess fluid and wrapped in saran wrap for autoradiography. The filters were checked with the monitor (Gieger counter) during the washing steps to avoid under or over washing.

In the darkroom, the filters were placed against x-ray films in the x-ray cassette. The films were exposed at –20$^{o}$C for 24–48 hours. The films were removed from the cassette while in the darkroom and processed using standard x-ray processing methods (developing in x-ray developer (Kodak) for 5 minutes, rinsing in water for 1 minute and fixing in a fixer (Kodak) with gentle agitation). Finally, the films were washed under running tap water for 15 minutes and air dried. Hybridization (black) signals were observed in the positive samples and controls, and no signals in the negative samples and controls. The unlabelled 1.6kb *C. elegans* unc17 (positive control) (probe) and PCR products (366bp and 1100bp) for *O. volvulus* showed positive signals. Similarly, the purified 1.6kb *C. elegans* unc17 hybridized intensely with 366bp and 1100bp $^{32}$P labelled probes which also hybridized to themselves and to each other (data not shown).

**3.1.10 Southern blotting**

In order to determine the genomic organization of the VAChT gene in worms and rat brain tissues, genomic DNA (20µg each) of *O. volvulus*, *H. contortus*, *C. elegans* or rat brain tissue was digested with BamH1, Hind111 or EcoR1 restriction enzymes according to procedures recommended by the manufacturers (Amersham). The digested

DNA samples were subjected to electrophoresis at constant voltage (50 volts) in 0.7% agarose gels in 0.5xTris buffered EDTA buffer (0.5xTBE) (45mM Tris borate, 1mM EDTA, pH 8.0). After electrophoresis, the DNA fragments were transferred to nylon membranes (Hybond N, Amersham) by capillary blotting as detailed in (3.9.5.2.1) above Sambrook *et al.,* (1989). The same probes as used for cDNA library screening were used. The filters were also processed in a similar manner as above (3.1.9.5.2).

### 3.1.11 Cloning of PCR fragments

Purified PCR products (366bp and 1100bp) were bluntly cloned into pMOSBlue vector and transformed into MOSBlue competent cells using the pMOSBlue blunt ended cloning kit (Amersham) according to the manufacturers' instructions as described below.

#### 3.1.11.1 Phosphate kinase (PK) reaction

For each PCR product to be cloned, a PK reaction was set up in a 1.5 ml eppendorf tube consisting of 1µl of 10x PK buffer, 0.5µl of 100mM DTT, 1µl of enzyme mix (supplied in the kit) plus 2µl of product (150ng of DNA) to be cloned and 5.5µl of $ddH_2O$ to make a final volume of 10µl. The reaction was incubated at 22$^{o}$C for 40 minutes.

The molar vector: insert ratio of 1:2.5 is optimal when using 50ng of vector. The tube was centrifuged briefly to collect the contents at the bottom of the tube. This ensures the kinase from the reaction remains at the bottom of the tube and ensures its complete inactivation. The reaction was heat inactivated by incubating at 75$^{o}$C for 10 minutes. Complete inactivation of the kinase enzyme is necessary to avoid vector background. The reaction was then cooled on ice for 2 minutes. The reaction must be chilled to avoid heat inactivation of the ligase in the ligation reaction. The reaction was briefly centrifuged to collect the condensate and proceed to the ligation protocol.

### 3.1.11.2 Ligation reaction

For a standard ligation reaction, 50ng of vector was ligated with the PK reaction. The ligation reaction was made up of 10µl of PK reaction product from above, 1µl of pMOSBlue vector (50ng/µl) and 1µl of T4 DNA ligase (4 Weiss units). The ligase was added last and mixed gently by stirring with a pipette tip. The reaction was incubated at 22$^{o}$C overnight.

### 3.1.11.3 Transformation

Luria broth agar plates containing 50µg/ml ampicillin and 15µg/ml tetracycline were used for plating competent cells. The tetracycline ensures that the selectable $F^{1}$ containing Lac ZΔ M15 is maintained and thus eliminates the background of non-recombinant white colonies which have lost the $F^{1}$. Competent cells (MOSBlue) (provided in the kit) were thawed on ice and mixed to evenly resuspend the cells. Microfuge tubes (1.5ml) were pre-chilled on ice and 20µl of competent cells pipetted into each pre-chilled tube. Ligation mix (1µl) was directly added to the cells and mixed by gentle stirring. The tubes were left on ice for 30 minutes. Then the cells were heat shocked for exactly 40 seconds in a 42$^{o}$C water bath. Cells were then placed on ice for 2 minutes. Room temperature SOC medium (80µl) was added to each tube. The tubes were incubated at 200-250 rpm at 37$^{o}$C for 1 hour.

For blue/white screening of recombinants, X-gal /1PTG plates were prepared by adding 35µl of 50mg/ml X-gal and 20µl of 100mM 1PTG per Luria agar antibiotic plate and left to soak for at least 30 minutes prior to plating. Each transformation (50µl) was spread on the L-agar plate. The plates were incubated when inverted overnight at 37$^{o}$C.

#### 3.1.11.4 Screening of recombinant colonies

The pMOSblue vector allows for blue-white screening with recombinant colonies appearing white when plated on X-gal and isopropyl-1-thio-β-D-galactosidase (1PTG) indicator plates. In addition to dark blue and white phenotypes, two other types were observed: (i) light blue, (ii) white with a blue center ("bullseye"). The frequency of these will depend on the length of incubation and the nature of the insert and is thought to result from the production of small amounts of the LaC Z α-peptide, possibly caused by ribosomal frame shifting, second site translation initiation or as α-peptide fusion protein. It has been shown however, that when screened these colonies contain insert with the same incident as observed with pure white colonies, that is, more than 75% contain inserts of the expected size when cloning purified insert. Only pure white colonies were screened in this study.

##### 3.1.11.4.1 Purification of recombinant inserts

White colonies (10) were individually inoculated into 2ml of LB/50μg/ml ampicillin and 15μg/ml tetracycline and incubated overnight at 37$^{o}$C and 300 rpm. Culture growths were harvested by centrifugation at 12,000xg, 4$^{o}$C for 5 minutes. Plasmid inserts were isolated from the pellets by alkaline lysis method as described briefly below. Cells were resuspended in buffer I (50mM Tris-Cl pH 7.5, 10mM EDTA, 100μg/ml RNAse A). After 5 minutes cells were lysed in buffer II (0.2M NaOH, 1% SDS). The mixture was neutralized by buffer III (2.55M potassium acetate pH 4.8). The DNA was precipitated by addition of 2 volumes of ice cold absolute ethanol (100%), centrifuged and thereafter pellet washed with 70% ethanol. After drying the pellet was resuspended in 50μl TE buffer (10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0).

Selected sites for endonuclease restriction upstream (SalI) and downstream (EcoR1) of the cloning site (EcoRV) were used in the reaction. In a 20µl reaction 5µl of the plasmid insert from each culture was digested with 1µl of each enzyme in appropriate buffer. The mixture was incubated at 37$^{o}$C for 3-4 hours after which the reactions were viewed on 1% agarose stained with Ethidium bromide (0.5µg/ml) following electrophoresis at 120 volts for 30 minutes. Clones/cultures containing the right sizes of the inserts (366bp and 1100bp) were selected for further use. Restriction of the plasmids with SalI and EcoRI yielded bands with additional 57 bp being flanking plasmid sequence between the two restriction sites.

**3.1.11.4.2 Direct colony PCR screening**

The presence of the appropriate insert and its orientation were also determined using direct colony PCR. White colonies (2) approximately 1mm in diameter were picked from each plate containing transformants using sterile pipette tips. The bacteria were transferred to 1.5 ml tubes containing 50µl of sterile water. The tubes were vortexed to disperse the pellet. The tubes were placed in boiling water for 5 minutes to lyse the cells and denature the DNAses. The tubes were centrifuged at 12,000 xg for 1 minute to remove cell debris. The supernatant (10µl) were transferred into fresh 0.5µl tubes and kept on ice until use. A master amplification mix was made up as follows per reaction; 27.5µl nuclease free water, 5µl of dNTPS, 1µl (5pmol/µl) of original PCR primer (upstream), 1µl (5pmol/µl) of original PCR primer (downstream), 5µl of 10X PCR buffer, 0.5µl (2.5U) Taq polymerase (Amersham). The above components were mixed together in a single tube using amounts corresponding to the number of reactions. Master amplification mixture (40µl) was added to each sample, mixed gently. The

reaction tubes were capped and placed in thermal cycler (Perkin-elmer 2400) with the following PCR program: 35 cycles of 94$^{o}$C for 1 minute, 55$^{o}$C for 1 minute and 72$^{o}$C for 2 minutes followed by one cycle at 72$^{o}$C for 5 minutes and then maintained at 4 $^{o}$C. PCR products (5µl) were analysed on 1% agarose gel containing Ethidium bromide (0.5µg/ml). DNA bands (366 bp and 1100 bp) were obtained for the appropriate ligation reactions.

The two DNA fragments isolated and sub cloned as above did not contain the 5' and 3' ends of the gene since the primers used for their amplification were designed from sequences located within the gene. To identify the missing 5' and 3' regions of *O. volvulus* VAChT gene, a rapid amplification of cDNA ends (RACE) was performed using a Marathon cDNA library of adult *O. volvulus* worms prepared from their mRNA as template as detailed below.

### 3.1.12 Messenger RNA extraction and purification

This was done using the Quick Prep$^{TM}$ micro mRNA purification kit (Amersham) as described by the manufacturer. Approximately 20-30 minutes before the worms were ready for the extraction, the kit was removed from storage at 4$^{o}$C and placed at room temperature. The extraction buffer was removed and placed at 37$^{o}$C until all the crystalline material was dissolved and then cooled to room temperature.

#### 3.1.12.1 Preparation for batch-washes

The oligo (dT)-cellulose slurry was gently swirled to obtained a uniform suspension. Aliquots of oligo (dT)-cellulose of 1ml were immediately pipetted into individual microcentrifuge tubes for each purification to be performed. The tubes were capped and left at room temperature.

### 3.1.12.2 Extraction of tissue samples

For extraction of tissue (up to 0.1g), the tissue (*O. volvulus* adult worms which had been kept in liquid $N_2$) was placed in a manual homogenizer and extraction buffer (0.4ml) was added to each 0.1g or less of material. The tissue was homogenized until it was a uniform suspension. The sample was diluted by adding 0.8ml of elution buffer (per purification) to the extract and mixed thoroughly. The sample was homogenized again briefly and the homogenate transferred to a sterile tube. Elution buffer (0.5ml per purification) was placed at 65$^{o}$C until needed.

### 3.1.12.3 Isolation of messenger RNA

#### 3.1.12.3.1 Binding step

A "cleared cellular homogenate" was prepared by centrifugation for 1 minute at high speed (10,000 xg), for each extraction sample. The tube containing oligo (dT)-cellulose (prepared above) was also centrifuged for 1 minute; this was done in parellel with the centrifugation of the sample. The buffer from the oligo (dT)-cellulose pellet was removed by using a 1ml pipette. The cleared cellular homogenate (1ml) was placed on top of the pellet of oligo (dT)-cellulose. The tubes were closed and inverted to resuspend the oligo (dT). The tubes were gently mixed by inverting manually for 3 minutes. The samples were centrifuged in a microcentrifuge at a maximum speed of 16,000 xg for 10 seconds. The tubes were removed from the centrifuge, the lids opened and the supernatant removed by pipetting.

#### 3.1.12.3.2 Washing steps

High salt buffer (1ml) was added to the tube. The lid was closed and the oligo (dT)-cellulose resuspended. The tube was placed in a centrifuge and spun for 10 seconds. The tube was removed from the centrifuge, lid opened and supernatant removed by pipetting.

The washing using high salt buffer for four more times as described above was repeated for a total of five washes. Low salt buffer (1ml) was added to the oligo (dT)-cellulose pellet and the lid closed. The resin was resuspended by inversion, or tapping the bottom of the tube. The tube was placed in a microcentrifuge and the resin pelleted by centrifugation at top speed for 10 seconds. The tube was opened to remove the supernatant by pipetting. Washing using low salt buffer was repeated one more time, exactly as described above, for a total for two washes. The resin was resuspended in 0.3ml of low salt buffer and the slurry transfered to a microspin column, placed in a microcentrifuge tube. The column was placed in the microcentrifuge and spun at high speed for 5 seconds. The column was removed from the microcentrifuge and the effluent discarded in the collection tube between each step if necessary for a total of three washes.

#### 3.1.12.3.3 Elution step

The column was placed in a sterile microcentrifuge tube and then placed in a microcentrifuge. Prewarmed elution buffer (200µl) was added to the top of the resin bed. The column was centrifuged at top speed for 5 seconds. A second aliquote of warm elution buffer was added as described above. The column was discarded and the tubes containing the eluted RNA placed on ice for precipitation.

#### 3.1.12.3.4 Precipitation of RNA

The mRNA was precipitated by adding 10µl of glycogen solution and 40µl of K acetate solution (1/10 volume) to the 400µl of sample. Chilled 95% ethanol (1ml) was added to the sample and placed at –20$^{o}$C for a minimum of 30 minutes. The precipitated mRNA was collected by centrifugation at 10,000xg in a microcentrifuge at 4$^{o}$C for 5 minutes. The supernatant was decanted and the tube inverted over a clean paper towel. The tube was gently tapped to facilitate the removal of excess liquid. The pellet was

redissolved into 20µl of elution buffer and kept at –80°C or in liquid nitrogen.

### 3.1.13 Preparation of *O. volvulus* double stranded cDNA

#### 3.1.13.1 First strand cDNA synthesis

In a sterile 0.5ml microfuge tube, 4µl of RNA sample (poly A+)(1µg), 1µl of cDNA synthesis primer (10µm) were combined, the contents were mixed by stirring with a pipette tip and tube spun briefly in a microfuge. The tube was incubated at 70°C for 2 minutes, cooled on ice for 2 minutes and then spun briefly to collect the contents at the bottom. The following reagents were added to the tube; 2µl of 5X First strand buffer, 1µl of AMV Reverse Transcriptase (20U/µl). The contents of the tube were mixed by gentle pipetting. The tube was spun briefly to collect the contents at the bottom. The tube was incubated at 42°C for 1 hour in an air incubator. The tube was placed on ice to terminate the first strand synthesis.

#### 3.1.13.2 Second strand cDNA synthesis

The following components were combined into a reaction tube; 10µl of first-strand reaction, 48.4µl of sterile water, 16µl of 5X second strand buffer, 1.6µl of dNTP mix (10mM) and 4µl of 20X second strand enzyme cocktail/mix. The contents were mixed thoroughly by gentle pipetting. The tube was spun briefly to collect the contents at the bottom. The tube was incubated at 16°C for 1.5 hours. Enzyme ($T_4$ DNA polymerase) (2µL) (10 Units) was added to the reaction mixture and thoroughly mixed by gentle pipetting. The tube was incubated at 16°C for 45 minutes. EDTA/glycogen mix (4µl) was added to terminate second strand synthesis. Phenol: chloroform: isoamyl alcohol (25:24:1) (100µl) was added to the tube. The tube was vortexed thoroughly and spun at high speed (14,000 xg) for 10 minutes to separate the phases. The top aqeous layer was

carefully transferred to a clean 0.5ml microcentrifuge tube. The interphase and lower phase were discarded. Chloroform: isoamyl alcohol (24:1) (100µl) was added to the aqeous layer and vortexed thoroughly. The tube was spun in a microcentrifuge at 14,000xg to separate the phases. The top aqeous layer was removed and placed in a clean 0.5ml microcentrifuge tube. Half volume (½) of 4M Ammonium acetate and 2.5 volumes of room temperature 95% ethanol were added to the tube. The mixture was vortexed thoroughly and spun immediately at 14,000xg at room temperature for 20 minutes.

The supernatant was removed carefully by aspiration and the pellet gently overlaid with 300µl of 80% ethanol. The tube was spun at 14,000xg for 10 minutes, the supernatant carefully removed. The pellet was air dried for approximately 10 minutes to evaporate residual ethanol. The pellet was dissolved in 10µl of water and stored at –20$^{o}$C. The double stranded (ds) cDNA (2µl) was analysed on 1.2% agarose Ethidium bromide stained gel with suitable DNA size markers and observed under UV light. Good ds cDNAs were obtained as was shown by the DNA smearing within the lanes from 3kb-0.5kb.

**3.1.13.3 Adaptor ligation of *O. volvulus* double stranded cDNA**

The ligation buffer (5x DNA ligation buffer) was allowed to completely thaw at room temperature for 30 minutes before use. The following reagents were combined in a 0.5µl micro centrifuge tube at room temperature in the order shown; 5µl of ds cDNA, 2µl of Marathon cDNA adaptor (20µM), 2µl of 5X DNA ligation buffer, 1µl of T4 DNA ligase (400 Units\µl). The tube was vortexed to mix the reagents and spun briefly in a micro centrifuge. The tube was incubated overnight at 16$^{o}$C. Next day, inactivation of

the ligase was done by heating at 70°C for 5 minutes. The adaptor ligated ds cDNA was diluted to concentrations suitable for RACE PCR (0.1µg/ml). The ds cDNA was diluted 1:50 and 1:25 with Tricine-EDTA buffer (provided in the kit). The diluted DNA was heated at 94°C for 2 minutes and immediately chilled on ice for 2 minutes, kept at –20°C until use.

The undiluted adaptor ligated ds cDNA was kept at –20°C until further use. The diluted Adaptor ligated double stranded cDNA was used as template for the RACE PCR reactions using the Clontech Advantage 2 PCR Amplification Kit (Amersham).

### 3.1.14 Rapid Amplification of cDNA Ends (RACE)

#### 3.1.14.1 RACE PCR to isolate the 5' end

To identify the 5′ end of *Onchocerca volvulus* putative vesicular acetylcholine transporter gene (*Ov*VAChT), a rapid amplification of cDNA ends was performed using a Marathon cDNA library of *O. volvulus* prepared above as the template. The sequence specific antisense oligonucleotide P8 (5′ GTCAGCGATCGCGTAGACGGA 3′) and the adaptor primer 1 (AP1) (5' CCATCCTAATACGACTCACTATAGGGC 3') (Clontech) were used. In a 50µl reaction volume, the PCR reaction comprised of 5µl of adaptor ligated cDNA (100ng), 5µl of 10X cDNA PCR buffer, 1µl of 50X dNTPS (10mM), 200mM of each primer and 1µl of 50X Advantage 2 Polymerase Mix (Clontech). The DNA template was denatured for 30 seconds at 94°C followed by 35 cycles of 94°C for 5 seconds and 68°C for 4 minutes, final extension at 68 °C for 3 minutes and then samples maintained at 4°C.

The PCR fragments were analysed by agarose gel electrophoresis revealing a PCR product approximately 1.4 kb of the expected size. To analyse the PCR product

further, nested PCR reactions with internal primers were done, revealing DNA band fragments of the expected sizes.

The PCR product (1.4kb) was purified from the agarose gel using the Sephaglas Kit (Amersham) as described above (3..1.8) and subcloned using the pMOSBlue Blunt Ended Cloning Kit (Amersham) as described above (3.1.11) and the nucleotide sequence was determined by sequencing as outlined below (3.1.15).

#### 3.1.14.2 RACE PCR to isolate the 3' end

Attempts to identify/isolate the 3' end of the gene were made using sense/forward primers designed from the conserved and non conserved regions, the latter were designed from the sequence of the already isolated, sequenced and cloned portions of the gene (1.4kb, 1.1kb and 0.366kb). Various sense/forward primers were used in combination with AP1 universal primer (Clontech) (antisense/reverse) primer during the PCR. The adaptor ligated cDNA of *O. volvulus* and other PCR components/reagents were used as indicated for the 5' RACE PCR. Additionally, various PCR conditions and reagents and sample concentrations were tried out, however, the 3' end could not be identified/amlified after many attempts/trials.

### 3.1.15 DNA sequencing

The PCR products subcloned from above were sequenced by Oswel Sequencing Company using T7 and U19 universal primers which are located upstream and downstream respectively of the EcoRV cloning site of the plasmid vector (pMOSBLUE). Both strands of the PCR products were sequenced, the nucleotide sequence was obtained and analysed to confirm its homology to known vesicular transmembrane transporters.

### 3.1.16 Restriction enzyme map construction and analysis

The longest coding gene fragment (1.2kb) was constructed and analyzed to

determine the restriction enzymes which could cut or not cut through the gene. The data is essential when determining the cloning sites within a vector. The Nebcutter computer analytical program was used for the construction and analysis. The enzymes which do cut through the gene and their locations and also those which do not have any sites to cut within the gene were indicated in the map. The results obtained from the computer analysis were confirmed in the laboratory by digesting the DNA with some of the commonly used restriction enzymes such as EcoR1, Sal1, BamH1, Hind111 and Kpn1.

### 3.1.17 Analysis of VAChT nucleic and amino acid sequences

The nucleotide sequences were translated into the corresponding amino acids automatically during blast searches. Nucleotide and amino acid homology searches were performed using Blastn and Blastx program commands respectively from various data genebanks such as NCBI, TIGR and SWISSPROT. Alignments of the homologous amino acid sequences were performed using ClustalX (Thompson *et al*., 1994) using default settings. Alignments were checked by eye and modifications made as required. The amino acids were identified as one (1) - letter codes according to the genetic code (Creighton, 1973) (Table 1). Names of the amino acids and chain termination codons are shown on the periphery of the circle. The first base of the codon is identified in the center ring; the second base of the codon in the middle ring; while the third base(s) of the codon are in the outer ring of the circle (Fig. 3).

#### 3.1.17.1 Phylogenetic analysis of VAChT amino acids sequences

Phylogenetic analysis of 25 homologous VAChT and VMAT sequences was undertaken using data generated from analysis of similarities and differences in the amino acids. Paup 4.0b1 (Swofford, 1998) was used to estimate genetic distances between individual sequences using the Kimura-2-parameter (K2: Kimura, 1980) distance metric

via neighbour-joining (NJ) analysis. Neighbour-joining analyses were based on K2 distances, and 1000 bootstrap analyses were performed by the NJ bootstrap method implemented in Paup. Although full length homologous VAChT and VMAT gene sequences are available for various animal species, the portions that corresponded with the isolated and sequenced portion of *O. volvulus* VAChT were used during the construction of the neighbour joining tree.

### 3.1.18 Sodium dodecyl sulphate-polyacrylamide and Western blotting

#### 3.1.18.1 Preparation of tissue total protein extracts

The adult worms or brain tissue were washed several times with cold distilled water and snap frozen in liquid nitrogen. Antigenic preparations of female *O. volvulus* and *H. contortus* adult worms and rat brain tissue of whole cell lysates were prepared by crushing the frozen samples using liquid nitrogen into powder using a mortar and pestle. The powder was added to ice cold PEM buffer containing PMSF proteinase inhibitor and spun at 1000xg for 30 minutes at 4$^{o}$C.

The supernatant (total proteins) were separated from the pellet (tissue debri) using a sterile pipette into new clean tubes and kept in 100µl aliquots at -80$^{o}$C. An equal amount of SDS sample reducing buffer containing 0.0625 M Tris (pH 6.8), 5 M urea, 4% (w/v) SDS, 10% (v/v) mercaptoethanol, 0.05% bromophenol blue and 10% glycerol, was added to each protein aliquot before further analysis. Protein mixtures were boiled for 10 minutes, cooled briefly and centrifuged at 13,000xg for 5 minutes prior to gel loading.

#### 3.1.18.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Four percent (4%) and 10% polyacrylamide gels were used as stacking and separating gels respectively. Two SDS-PAGE gels were run under identical conditions on one apparatus. Electrophoresis was done at 100 V for 1 hour. The conducting buffer was

0.025 M Tris-HCl (pH 8.3) containing 0.192 M glycine and 2% SDS. Protein standards (Biorad) were used to determine the sizes of the various proteins in the total/crude protein extracts. After electrophoresis, one gel was stained with Coomassie Brilliant Blue and eventually destained with destaining buffer (Appendix 7.1). The stained gel was scanned to obtain a permanent record. The proteins in the equivalent gel were electrophoretically transferred onto nitrocellulose paper (Hybond C extra membrane) (Amersham) for immunoblotting.

**3.1.18.3 Western blotting**

Western blot analysis was carried out as described in Harlow and Lane (1988). The SDS PAGE proteins were transferred to nitrocellulose (Hybond C-extra membrane) (Amersham) utilizing the mini-Transblot cell (Biorad) and the protocol for the apparatus. The electroblot transfer was performed at 100 V for 1 hour. After protein transfer, the nitrocelluloses were rinsed with phosphate buffered saline (PBS) (pH 7.3). After rinsing, the membranes were incubated with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) overnight at 4ºC to block non-specific binding.

The primary antibody was an affinity isolated rat anti-vesicular acetylcholine transporter (VAChT) antibody (2.3ug/ml) (Sigma-Louis, MO). The secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit IgG (whole molecule) (Sigma-Louis, MO). The diluted primary (1:1000) and secondary (1:10000) antibody contained 1% (w/v) BSA and 0.1% Tween-20. The blots were washed with PBS containing 0.4% Tween-20, for 3 x 15 minutes following 2 hours incubation with the primary or secondary antibody at room temperature while shaking. A colour substrate reagent (bromochloroindolyl phosphate/nitro blue, tetrazolium solution) (BCIP/NBT) (Sigma-Louis, MO) was used to visualize the reactions within 10-30 minutes. The membranes

were eventually washed in distilled water for 10 minutes while rocking to stop the reactions and eventually air-dried and stored at room temperature.

### 3.2 *In vitro* evaluation of vesamicol inhibitory effects in *H. contortus*

#### 3.2.1 Parasite collection

Twenty one (21) fresh abomasums of goats were obtained from Kampala City Council (KCC) abattoir (one abomasum at a time), immediately after animal slaughter and transported to the Department of Parasitology and Microbiology, Molecular Biology Laboratory. In the laboratory, the abomasums were opened up longitudinally using a pair of sterile scissors and the worms were isolated from the abomasal contents and/or walls using blunt forceps. All the worms present in the abomasum were isolated and placed in pre-warmed physiological saline at 37$^{o}$C. Basing on the morphological features of the worms that is, the barber's pole appearance due to the winding of the white uterus and ovary to the blood filled intestines, the female *Haemonchus contortus* adult worms were identified and used in this study. Six (6) worms (all females) were used for each of the inhibitor dilutions and the same number of worms were used for positive and negative control group

#### 3.2.2 Parasite maintainance *in vitro*

The isolated worms were kept in pre-warmed physiological saline (Appendix 7.2), at 37$^{o}$C as the isolation was being done. The worms were washed in physiological saline to free them of any debris. The worms were incubated in physiological saline for 15 to 30 minutes, so that they get acclimatized to the environment before allocation to inhibitor /drug treatment.

The worms were transferred into a 24 well plate (Falcon) and maintained individually in 1.8ml of physiological saline containing appropriate inhibitor

concentration. Streptomycin (200µg/ml) was incorporated into the medium, to prevent bacterial infection during the experimentation period.

### 3.2.3 Preparation and administration of inhibitor dilutions

The compound L(-)-Vesamicol hydrochloride was obtained from Research Biochemicals International (Natick, MA, USA). The compound was reconstituted as a 20mM stock in absolute Dimethyl sulfoxide (DMSO) (solvent) and diluted to working concentrations in physiological saline. Negative controls contained DMSO at concentrations ranging from 0.5 to 5.0% v/v, equivalent to the DMSO in the corresponding inhibitor dilutions, to ensure that there were no artifactual effects of DMSO on the worms, while positive controls contained levamisole (a renowned cholinergically active antihelmintic), at the same concentration as that of vesamicol. The worms were incubated in vesamicol dilutions of 0.1mM, 0.2mM, 0.3mM, 0.4mM, 0.5mM, 0.6mM, 0.7mM, 0.8mM, 0.9mM or 1.0mM. All experiments were performed in duplicate and repeated twice.

### 3.2.4 Measurement of worm motility

The worms were incubated in the working inhibitor dilutions at 37$^{o}$C in a water bath and assessments of worm motility were made at times; 0, 30, 60, 90, 120, 150 and 180 minutes of incubation. Each treated group consisted of 6 worms (all females). The positive and negative controls also had the same number of worms. The worms were observed visually for their motility and each worm was observed continuously for 20 seconds during each assessment. The motile and immotile worms were counted and recorded and eventually added together for each corresponding inhibitor concentration and incubation period.

### 3.3 Data analysis

A time-response graph showing the effect of three representative concentrations of vesamicol (0.1mM, 0.5mM and 1mM) at the different periods of incubation, on motility of the worms was fitted by non-linear regression analysis, using the computer program, Graphpad prism (Graphed Software, San Diego, CA, USA). A response was expressed as percent inhibition of the initial response (control motility) and each point plotted as the percent inhibition of data obtained from the successive experiments. Percent inhibition (P.I.) was obtained as follows,

$(P.I) = N_m\text{-}N_iT/N_m) \times 100$

where;

$N_m$ = Initial number of motile worms in the test group.

NiT = Number of immotile worms in the test group

Values of Cmax (the concentration that causes the maximum inhibition in motility) and Emax (the maximum response) were determined from the time response graph.

Analysis of variance in the mean percentage inhibition of worm motility caused by the various vesamicol concentrations at the different periods of incubation was carried out using 2-Way ANOVA from the computer program Graph pad prism (Graphed Software, San Diego, CA, USA) to determine whether the variations in the mean percent inhibition of worm motility were statistically significant.

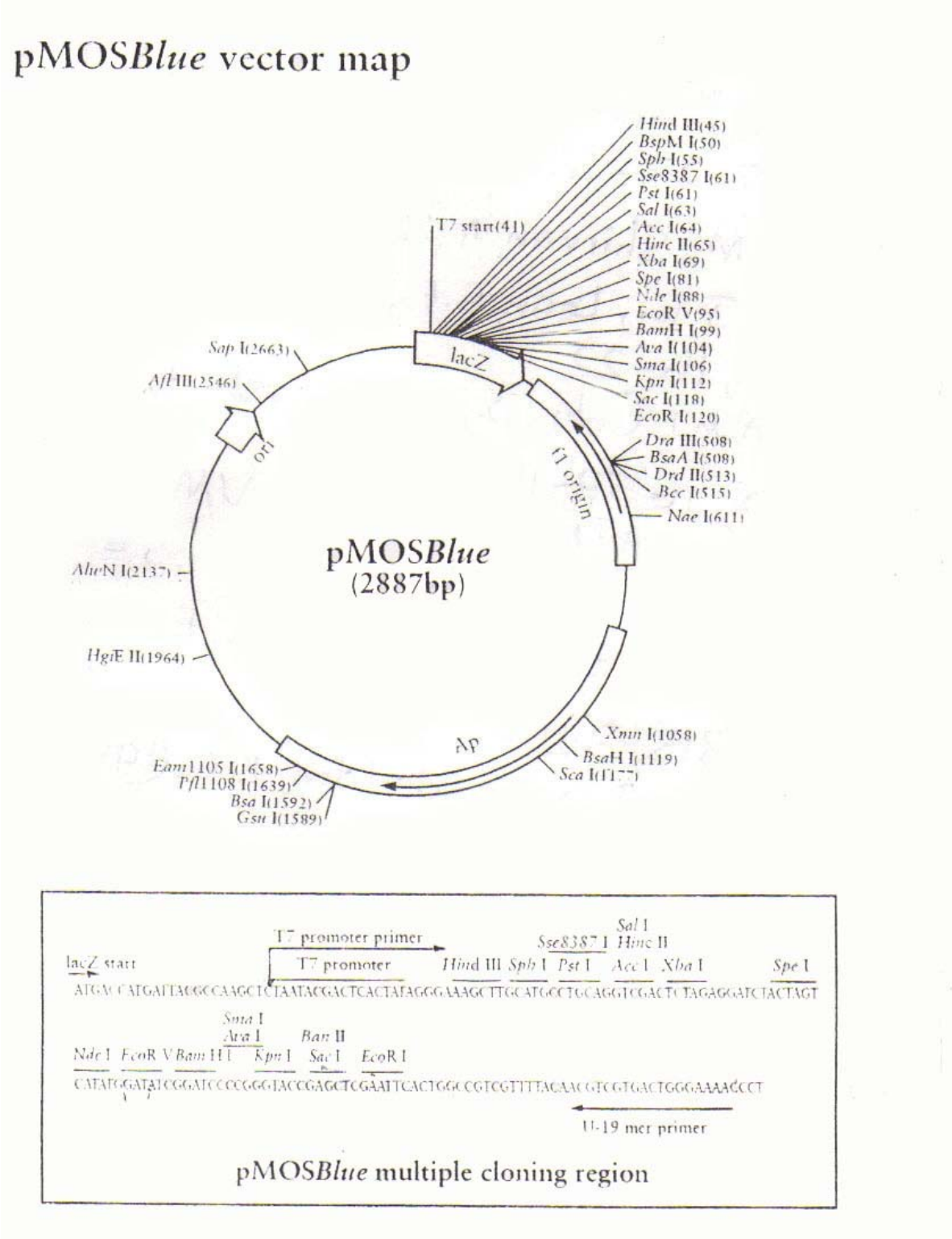
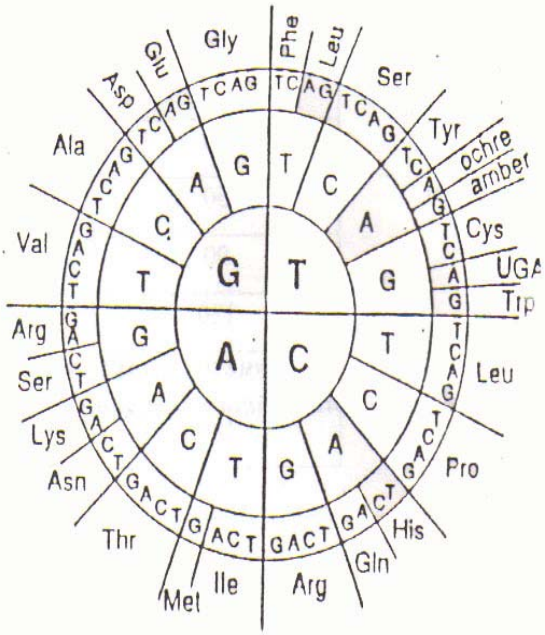



**Figure 2: Map of pMosBlue plasmid vector showing the multiple cloning region**
The region is flanked by $T_7$ promoter and U-19mer sequences up and downstream.

**Table 1: Three (3) and one (1) letter amino acid code**

| Amino acid | 3-letter code | 1-letter code |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartate | Asp | D |
| Cysteine | Cys | C |
| Glutamate | Glu | E |
| Glutamine | Gln | Q |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

**Figure 3: Genetic code**



# CHAPTER FOUR

# RESULTS

## 4.1 Cloning and characterization of *O. volvulus* VAChT gene

### 4.1.1 Primer design

Various oligonucleotide primers were designed and used for the PCR since the VAChT gene structure in *O. volvulus* was not known and an intron could be present between the nucleotide binding sites. Alignment of the amino acid sequences of the putative vesicular acetylcholine neurotransmitter transporter from *C. elegans* (unc17), and two rat vesicular monoamine transporters (VMATs) (rVMAT1 and rVMAT2) (Alfonso *et al*., 1993), revealed five regions that are strongly conserved among species. The amino acid stretches LLLDNML, LFASKA, LAFLE, CFGIALI and SVYAIAD (Fig. 6 in bold) were used for designing 12 oligonucleotides (Primers 1-12) (Table 2) which would be likely to bind to the *O. volvulus* putative vesicular acetylcholine transporter gene.

When an amino acid was not absolutely conserved or when various codons had been used to encode the same amino acid, preference was given to the *C. elegans* nucleotide since it was coding for the VAChT gene and it is a nematode with A-T rich nucleotide composition like *O. volvulus* for the 8 primers (P1-P8) (Table 2). In addition, four degenerate primers (P9-P12) (Table 2) were also included to increase the chances of amplifications under less stringent conditions such as low annealing temperatures.

Amplifications from genomic DNA or cDNA libraries of *O. volvulus* with 2 primer sets P1 or P4 (forward) and P8 (reverse) revealed PCR products 1100bp and 366bp respectively of the expected molecular weight according to the regions of primer

design (Fig. 4: lanes 2&3). Authenticity of the amplified DNA fragments was further verified by cloning and sequencing. Pearson TFasta-assisted analysis revealed that the two PCR fragments encoded amino acids that are conserved in other VAChTs and VMATs and they overlapped at their 3' ends as expected since the same reverse primer (P8) was used for both PCR amplifications.

Since the efforts to isolate the complete *O. volvulus* VAChT from the cDNA libraries and genomic DNA by screening and PCR met no success, it was decided that the complete coding sequence be obtained by 3' and 5' rapid amplification of cDNA ends using 3' and 5' RACE kit (Clontech). Adult worm *O. volvulus* cDNA was synthesized from polyadenylated (A+) RNA using the universal primer containing poly (dT) sequences and an adaptor tail (Clontech).

| NAME | TYPE | SEQUENCE | Tm ($^0$C) |
|---|---|---|---|
| P1 | Forward | CTACTACTCGACAATATGCTC | 60.0 |
| P2 | Forward | CTCTTCGCTTCAAAAGCT | 52.0 |
| P3 | Reverse | AGCTTTGAAGCGAAGAG | 52.0 |
| P4 | Forward | CTCGCCTCCCTCGAG | 52.0 |
| P5 | Reverse | CTCGAGGAAGGCGAG | 50.0 |
| P6 | Forward | TGCTTCGGAATCGCCTTGATC | 64.0 |
| P7 | Reverse | GATCAAGGCGATTCCGAAGCA | 64.0 |
| P8 | Reverse | GTCAGCGATCGCGTAGACGGA | 68.0 |
| P9 | Forward | CTNCTNCTNGAYAAYATGCTN | 42.0 |
| P10 | Forward | CTNGCNTCYCTNGAR | 32.0 |
| P11 | Reverse | YTCNAGRGANGCNAG | 32.0 |
| P12 | Reverse | RTCNGCRATNGCRTANACNGA | 42.0 |

**TABLE 2: Primers designed from the conserved regions of unc17 and 2 rat monoamine transmitter transporters**

Primers 1-8 are specific with definite bases and high melting temperatures, while primers 9-12 are degenerate, with variable bases and low melting temperatures.

Additional primers (A-J) (Table 3) which are specific for the *O. volvulus* VAChT gene were designed from the nucleotide sequence of the initially isolated 366bp PCR fragment. The regions with high G-content were used for primer design so as to have primers with high melting points (Tm) with could be used with AP1 (univerval) primer (Clontech) for touch down PCR or amplifications at high annealing temperatures. Primer K (PK) in Table 3 represents the spliced leader (SL) sequence which occurs at the 5' end of genes in nematodes. This was designed with the aim of pulling out the 5' end of the gene including the untranslated base sequences at the beginning of the gene.

| **NAME** | **TYPE** | **SEQUENCE** | **Tm ($^0$C)** |
|---|---|---|---|
| Primer A | Forward | TCCGTCTACGCGATCGCTGACATT | 59.0 $^0$C |
| Primer B | Forward | TTACGGATCCGTCTA CGCGATCGC | 60.0$^0$C |
| Primer C | Reverse | GTCAGCGATCGCGTAGACGGA TCC | 62.5 $^0$C |
| Primer D | Reverse | GCGATCGCGTAGACGGATCCGTAA | 60.8 $^0$C |
| Primer E | Forward | ATGCCAGACACTCCTGGCTGGTTG | 60.8 $^0$C |
| Primer F | Reverse | CAAGCCAGGAGTGTCTGGCAT | 56.3 $^0$C |
| Primer G | Forward | GTGATGCAACTTGTCATCCCGCTCTCT | 61.2 $^0$C |
| Primer H | Forward | GGGTTGGCTATGGAGGGAATCGCGTGT | 64.2 $^0$C |
| Primer I | Reverse | AGAGAGCGGGATGACAAGTTGCATCAC | 64.2 $^0$C |
| Primer J | Reverse | ACACGCGATTCCCTCCATAGCCAACCC | 64.0 $^0$C |
| Primer K | Forward | GGTTTAATTACCCAAGTTTGAG | 49.2 $^0$C |

**TABLE 3: Specific primers for *O. volvulus* VAChT gene or spliced leader sequences**
Primers (A-J) were designed from the isolated and sequenced *O. volvulus* 366 PCR fragment. Primer K is the spliced leader sequence (SL1) which occurs at the beginning of all nematode genes.

The 5' RACE PCR on the *O. volvulus* adult worm adaptor ligated cDNA pool template, using the universal (AP1) (forward) and P8 (reverse) primers yielded a 1.4kb 5' gene fragment (Fig. 4; lane 1). Nested PCR using universal (AP2) (forward) (overlaps

with AP1) and PJ (reverse) (internal to P8) primers amplified a gene fragment approximately 1.3kb (data not shown), of the expected molecular weight size.

The 3' RACE PCR with universal (AP1) (reverse) and any of the forward primers in tables 2 and 3 above did not reveal any representative amplification. The majority of the reactions resulted into smears or no amplification products. Occasionally the PCR products could not be confirmed by nested PCR using internal primers. Even upon sequencing, blast analysis and sequence alignment, and the 3' PCR products did not show the anticipated homology in the overlapping regions and therefore could not be analyzed further.

The DNA sequencing of the longest 5' RACE PCR product prompted the design of another set of primers (Ann1-6) (Table 4), the majority of which were forward primers to make further attempts to obtain the 3' end although this still met no success. Primer Ann2 (forward) which was designed from the ATG start codon of the gene was used together with P8 (reverse) primer to confirm the coding sequence (1.2kb) (Fig 4; lane3) of the gene as revealed by sequencing. Other primers were designed from the conserved poly-A tail sequence (Ann 7) while primers (Ann8-Ann11) were designed from the 3' end sequence of *C. elegans* unc17 (Table 4).

Further attempts were done to obtain the 3' end by designing more primers with higher melting temperatures and GC-content (forward) (Table 5). Specific bases were added to the poly-T primers (Table 5) to increase their melting temperatures so as to enable amplifications at higher annealing temperatures. The primer sets were used with various DNA or RNA template sources.

| NAME | TYPE | SEQUENCE | Tm ($^0$C) |
|---|---|---|---|
| Ann1 | Forward | TCCGTCTACGCGATCGCTGACATT | 59.0 $^0$C |
| Ann2 | Forward | ATGGGCTTCAACGTGCCCGTCATCCAA | 70.7$^0$C |
| Ann3 | Reverse | GTCAGCGATCGCGTAGACGGA TCC | 62.5 $^0$C |
| Ann4 | Reverse | GCGATCGCGTAGACGGATCCGTAA | 60.8 $^0$C |
| Ann5 | Forward | ATGCCAGACACTCCTGGCTGGTTG | 67.6 $^0$C |
| Ann6 | Reverse | CAAGCCAGGAGTGTCTGGCAT | 56.3 $^0$C |
| Ann7 | Reverse | TTTTTTTTTTTTTTTTTTTTTTTTT | 50.4 $^0$C |
| Ann8 | Forward | GGGTTGGCTATGGAGGGAATCGCGTGT | 64.2 $^0$C |
| Ann9 | Reverse | AGAGAGCGGGATGACAAGTTGCATCAC | 64.2 $^0$C |
| Ann10 | Reverse | ACACGCGATTCCCTCCATAGCCAACCC | 64.0 $^0$C |
| Ann11 | Forward | GGTTTAATTACCCAAGTTTGAG | 49.2 $^0$C |

**Table 4: Primers designed from known VAChT or poly-A tail sequences**
Primers (Ann1-Ann6) were designed from the amplified and sequenced 1.2kb *O. volvulus* PCR fragment. Primer (Ann7) was designed from the conserved poly-A tail sequence while primers (Ann8-Ann11) were designed from the 3' end sequence of *C. elegans* unc17.

| NAME | TYPE | SEQUENCE | Tm ($^0$C) |
|---|---|---|---|
| A1 | Forward | ATGGGCTTCAACGTGCCCGTCATCAACCGAGAC | 73.2 $^0$C |
| A2 | Forward | CTCTGCCCTCCTCGAGCCCACCATCACAACCTG | >75.0$^0$C |
| A3 | Forward | CTACTACTCGACAATATGCTCTACATGGTCATT | 65.8 $^0$C |
| A4 | Reverse | GTCAGCGATCCCGTAGACGGA | 63.7 $^0$C |
| A5 | Reverse | GTCAGCGATCGCGTAGACGGATCCGTAAACCCAAA CG | >75.0$^0$C |
| A6 | Reverse | GCGGCCGCTTTTTTTTTTTTTTTTTTTTTTTTTTT | 60.8 $^0$C |
| A7 | Reverse | TTCTAGAATTCAGCGGCCGCTTTTTTTTTTTTTTTTT TTTTTTTTTTT | 65.3 $^0$C |
| A8 | Forward | CTAATACGACTCACTATAGGCC | 58.4 $^0$C |

**Table 5: Primers designed from *O. volvulus* VAChT 1.2kb fragment or poly-A tail**
Primers (A1-A5) with high melting temperatures and (A8) with moderate melting temperature were designed from the 1.2kb fragment In addition to specific bases, poly T tails were included for primers A6 and A7 primers.

### 4.1.2 Isolation of *O. volvulus* VAChT gene by PCR

Initually a 366bp fragment (Fig. 4; lane 1) could be isolated from *O. volvulus* genomic DNA and cDNA libraries using primers, P4 and P8 (Table 2). This was sequenced and found to match an internal section of the *C. elegans* unc 17 gene when blasted against known DNA sequences in the gene databases. Subsequently, a 1100bp fragment (Fig. 4; lane 2) overlapping the 366bp fragment was isolated from both template DNA sources using primers, P1 and P8 (Table 2) and matched with the unc 17 of *C. elegans*.

The 5' RACE PCR product (OVG1400) (1.4kb) (Fig. 4; lane 4) was amplified from the adaptor ligated cDNA pool prepared from the mRNA of the adult *O. volvulus* worms.. This fragment contains the 5' untranslated region (UTR) (122bp) long and the beginning of the 5' end of the *O. volvulus* vesicular acetylcholine transporter. The latter was confirmed by PCR using forward primer (Ann2) (Table 4) designed from the sequences at the beginning of the gene, which together with the same reverse primer (P8) amplified a PCR fragment (1.2kb) in open reading frame (ORF) (Fig.4; lane 3). The initially amplified internal PCR fragments (366bp and 1100bp) (Fig.4; lanes 3&4) were also confirmed in the subsequent PCR amplifications whereby the 1.4kb or 1.2kb PCR fragments were used as the templates and also by sequence alignments.

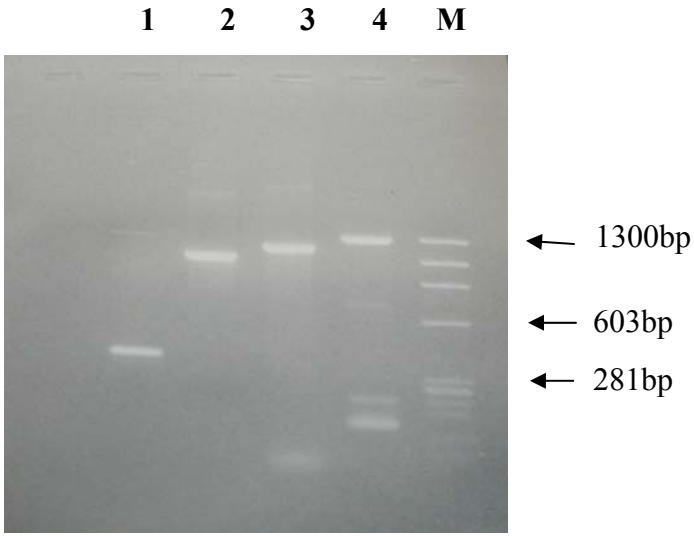



**Figure 4**: **Agarose gel showing various PCR amplifications of *O. volvulus* VAChT**
Lane 1: PCR product (366bp) with P4 and P8 primers, Lane 2: PCR product (1100bp) with P1 and P8 primers, Lane 3: PCR product (1200bp) with Ann2 and P8 primers, Lane 4: PCR product (1400bp) with AP1 and P8 primers, Lane M: DNA size marker (ΦX 174 DNA marker Hae 111 digest–Sigma DO672). Band sizes (bp) for the marker are indicated on the right.

Several primer pairs designed from various conserved and/or known sequences of the gene were used, since the gene structure was unknown and introns could be present. Primer AP1 is a universal primer which hybridizes with the adaptor used in the construction of the cDNA pool. The above primer pairs amplified DNA fragments of the expected band sizes at various PCR conditions, confirming the presence of the VAChT gene in *O. volvulus.*

### 4.1.3 Verification of the *O. volvulus* VAChT gene by sequence analysis

#### 4.1.3.1 Nucleotide and deduced amino acid sequence analysis

Sequencing of both strands of the longest PCR product (1.4kb) using the sequenase kit (Amersham) and corresponding amino acid translation using Bioedit or ClustalX showed an open reading frame comprising of 1212 bases/nucleotides and 404

amino acid residues contained in the 5'end of the gene (Fig.5) and an untranslated 5' end region of 122 bases(data not shown). The 3' end of the gene is still missing since a stop codon or a poly A tail was not encountered as the reverse primer used with success was designed from the last conserved region which is located within the gene. The PCR fragments (1.2kb, 1.1kb, 0.366kb) isolated from genomic DNA of adult *O. volvulus* worms and cDNA template sources were similar by agarose gel (same size) and sequencing (same nucleotides) (not interrupted by introns). Whether this organization of gene continuity is common throughout the entire gene is unknown since the 3' end is still missing from all template sources.

Basing on the size of *C. elegans* unc17 gene homologue and western blotting of the rat VAChT, the missing fragment of the *O. volvulus* VAChT gene is speculated to be approximately 500bp.

#### 4.1.3.2 Restriction map analysis of the *O. volvulus* 1.2kb VAChT sequence

The DNA sequence (nucleotides 1-1212) was analysed using the Nebcutter computer program which generates a genetic map indicating the restriction enzymes which cut through the DNA sequence. Amongst the commonly used restriction enzymes, EcoR1, Mlu1, Sal1 and EcoRV do not cut through the gene whereas Hind111 and BamH1, each cuts through the fragment once at variable sites/positions (data not shown). This data was confirmed by digesting the 1.2kb DNA fragment with the appropriate enzymes which revealed the predicted computer results (data not shown).

```
atgggcttcaacgtgcccgtcatcaaccgagactcggagatcctcaaagcggacgccaaa
  M  G  F  N  V  P  V  I  N  R  D  S  E  I  L  K  A  D  A  K
aagtggcttgagcagcaggataatcagaagaaatgcgtcctggtgatagtctccatagcc
  K  W  L  E  Q  Q  D  N  Q  K  K  C  V  L  V  I  V  S  I  A
ctactactcgacaatatgctctacatggtcattgtcccaattattccgaaataccttcgg
  L  L  L  D  N  M  L  Y  M  V  I  V  P  I  I  P  K  Y  L  R
gacattcataactaccaggtgaccttcgagggataccacaatgagacaagccagcttgcg
  D  I  H  N  Y  Q  V  T  F  E  G  Y  H  N  E  T  S  Q  L  A
aacgggacctatctggtaagagaggtcggcggaaggattaacttttggatgaggagctg
  N  G  T  Y  L  V  R  E  V  G  G  R  I  N  F  L  D  E  E  L
gaattgggatggctcttcgcttcaaaagctttgctgcaaattttgtgaatccgttttcg
  E  L  G  W  L  F  A  S  K  A  L  L  Q  I  F  V  N  P  F  S
ggatatattatcgatcgagttggatacgagattccgatgattttggggctatgcaccatg
  G  Y  I  I  D  R  V  G  Y  E  I  P  M  I  L  G  L  C  T  M
tttttctccacggctatatttgctcttggaaagagctatggagttttgctttttgcaaga
  F  F  S  T  A  I  F  A  L  G  K  S  Y  G  V  L  L  F  A  R
tcgttgcagggcttcggctcagcgttcgccgatacttccggtcttgccatgatcgccgat
  S  L  Q  G  F  G  S  A  F  A  D  T  S  G  L  A  M  I  A  D
cgattcaccgaagaaaacgagagatcagcggcacttggaatcgccttagcctttatctcg
  R  F  T  E  E  N  E  R  S  A  A  L  G  I  A  L  A  F  I  S
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  F  G  C  L  V  A  P  P  F  G  S  V  L  Y  S  L  A  G  E  P
gtacctttcctgattctgtcatttgtctgtttggctgatgctattgcagttttatggtg
  V  P  F  L  I  L  S  F  V  C  L  A  D  A  I  A  V  F  M  V
atcaatccccatcggagaggcactgattctcatggagagaaggttcaaggaaccccaatg
  I  N  P  H  R  R  G  T  D  S  H  G  E  K  V  Q  G  T  P  M
tggcgtctcttcatggatcccttcatcgcttgctgctccggagccctcatcatggccaac
  W  R  L  F  M  D  P  F  I  A  C  C  S  G  A  L  I  M  A  N
gtctctctcgccttcctcgagcccaccatcacaacctggatgtccgaaatgatgccagac
  V  S  L  A  F  L  E  P  T  I  T  T  W  M  S  E  M  M  P  D
actcctggctggttggtcggagtcatctggctcccaccattctttccacacgttctcgga
  T  P  G  W  L  V  G  V  I  W  L  P  P  F  F  P  H  V  L  G
gtctatgtgaccgtcaaaatgctcagggcgttcccccaccacacatgggcgattgctatg
  V  Y  V  T  V  K  M  L  R  A  F  P  H  H  T  W  A  I  A  M
gttgggttggctatggagggaatcgcgtgttttgcaatcccctataccacatcggtgatg
  V  G  L  A  M  E  G  I  A  C  F  A  I  P  Y  T  T  S  V  M
caacttgtcatcccgctctcttttgtatgcttcggaatcgccttgatcgacacctcgctc
  Q  L  V  I  P  L  S  F  V  C  F  G  I  A  L  I  D  T  S  L
ctcccgatgcttgtacaccttgtggacacccgccacgtttcggtttacggatccgtctac
  L  P  M  L  V  H  L  V  D  T  R  H  V  S  V  Y  G  S  V  Y
gcgatcgctgac
  A  I  A  D
```

**Figure 5: Nucleotide and amino acid sequences of the partial *O. volvulus* VAChT**

The translation and alignment were done using the Bioedit computer program. Introns are absent as an open reading frame was obtained from both the genomic and cDNA template sources by PCR. Capital letters represent amino acids and small letters represent the DNA bases by single letter standard coding.

#### 4.1.3.3 Homology analysis of *O. volvulus* VAChT amino acid sequences

The similarity of the isolated fragment (1.2kb) to previously sequenced homologues was analyzed by multi-alignment sequence analysis using clustalX computer program against National Center for Biotechnology Institute (NCBI) database (Fig.6; Appendix 8.6). The greatest degrees of sequence similarity were found with the putative vesicular acetylcholine transporters from *C. elegans* (unc17) (98%), *Drosophila melanogaster* (DroMEVAChT) (56%), *Anopheles gambiae* (ENSANGP00000001263) (54%) and *Torpedo californica* (TorVAChT) (55%) as well as to the acetylcholine transporters sequences from other *Torpedo species* that have been reported (55%) (Fig. 6 & Appendix 8.6), rat VAT (49%), human VAT (49%) and bovine VAT (38%) (Appendix 8.6). Somewhat less but still substantial resemblance to the human and rat monoamine transporters (MAT) (36-39%) was also identified (Appendix 8.6). Less pronounced similarity (22%) was also displayed to a class of bacterial transporters which confer drug resistance (Appendix 8.6).

The *Ov*VAChT amino acid sequence varied from the *C. elegans* unc17 homologue by substitution of three amino acids (Fig.6). The substitution positions 219 (lysine to glutamate), 384 (arginine to methionine) and 386 (valine to glycine) in *O. volvulus* VAChT reflected functional changes.

The predicted partial protein (*Ov*VAChT) from the amino acid sequence of the longest coding cDNA (1.2kb) forms 11 transmembrane domains (Fig. 6), however, it still lacks the 3' end and thus still missing the 12$^{th}$ transmembrane which is recorded in other VAChTs and MATs. The cloned gene fragment is approximately 75% of the complete gene of the *C. elegans* unc17 (1.6kb) homologue, on this basis, 25% or less of the onchocerca gene remains to be isolated. The 11 putative transmembranes which are

present in this gene are characteristic of the known vesicular neurotransmitter transporters. In particular, aspartic acid residues in transmembranes 1, 6, 10 and 11 that may be required for substrate binding and cationic amine/H antiport (Erickson and Eiden, 1993) are also conserved in *O. volvulus* VAChT (Fig. 6).

Like other known VAChTs and MATs a large luminal loop is predicted to occur between the first two transmembane domains (Fig. 6). The loop contains two potential sites for N-linked glycosylation. These observations further suggest that this protein belongs to the family of vesicular neurotransmitter transporters/H antiporters with 12 transmembrane domains. The highest divergence between the onchocerca VAChT protein sequence and those of the homologous aligned transporters lies in the large luminal loop and N-terminal end sequence. This divergence is anticipated in the C-terminal end based on information from known homologous complete genes (Erickson and Eiden, 1993).

```
O.volv.VAChT    1 ---------M GFNVPVINRD SEILKADAKK WLEQQDNQKK CVLVIVSIAL LLDNMLYMVI V
C.eleg.UNC17    1 ---------. .......... .......... .......... .......... .......... .
Drosoph.VAChT   1 --------MA S.QI....LE VREV.DIVWE KIQEPV..RR LI........ .......... .
A.gamb.VAChT    1 ---------- ---L.I..LE PSEV.EIFWT KVKEPQS.R. LI........ .......... .
Torca           1 MGVTMAVGLA KAAMGK.SSA IGERSKRISG AMNEPRRKR. IL....C..M .......... .
                                                                      TM1
O.volv.VAChT   62 PIIPKYLRDI HNYQVTFEGY HNETSQLANG TYLVREVGGR INFLDEELEL GWLFASKALL Q
C.eleg.UNC17   62 .......... .......... .......... .......... .......... .......... .
Drosoph.VAChT  62 ....D...E. GSFDDGPTPP PLRDN----- -ITGKIIPVH HDHHGQDSAS .I......IV .
A.gamb.VAChT   62 ....D...Y. GTW--GP.EP Y.MSA----- -PTTVFTPHT HSHHGQDSAT .I......IV .
Torca          62 ....N..ET. RT.KLVYITT PSNGTNGSLL NSTQ.A.LE. NPNAN.DIQI .V......I. .
                                                                                  TM2
O.volv.VAChT  123 IFVNPFSGYI IDRVGYEIPM ILGLCTMFFS TAIFALGKSY GVLLFARSLQ GFGSAFADTS G
C.eleg.UNC17  123 .......... .......... .......... .......... .......... .......... .
Drosoph.VAChT 123 LM......GL ..KI..DL.. MI..TI.... ..V..C.S.. S..F...... .V.......A .
A.gamb.VAChT  123 LM......AL ...I..DL.. MV..II..L. .MV..C.R.. SM.F...... .V.......A .
Torca         123 LLS...T.TF ......D..L LI..TI.... .IT..F.E.. A..FA...... .L........ .
                                      TM3                                       TM4
O.volv.VAChT  184 LAMIADRFTE ENERSAALGI ALAFISFGCL VAPPFGSVLY SLAGEPVPFL ILSFVCLADA I
C.eleg.UNC17  184 .......... .......... .......... .......... ....K..... .......... .
Drosoph.VAChT 184 .......... .....Q.... .......... ......GA.. QF..KE.... ..AL...L.G L
A.gamb.VAChT  184 .......... .A..TK.... .......... ......GA.. QF..KE...V ..ALIS.I.G F
Torca         184 I.....KY.. .S..TQ.... ........S. ......G... QF..KW.... V......L.G .
                                      TM5                                       TM6
O.volv.VAChT  245 AVFMVINPHR RGTDSHG-EK VQGTPMWRLF MDPFIACCSG ALIMANVSLA FLEPTITTWM S
C.eleg.UNC17  245 .......... .......-.. .......... .......... .......... .......... .
Drosoph.VAChT 245 MLLL.MK.VK EAMKQSKDVQ D.VI.I...L ...Y..V.A. .MT.S..A.. ......SL.. E
A.gamb.VAChT  245 MLLL.MK.IK EQLADRQEVR APSV.I...L ...Y..V.A. ..M.S..A.. ......SL.. E
Torca         245 LLL..VT.FA SR.REN---M L....IYK.M I..Y..VVA. ..TTC.IP.. ....SN.. K
                                                                      TM7
O.volv.VAChT  306 EMMPDTPGWL VGVIWLPPFF PHVLGVYVTV KMLRAFPHHT WAIAMVGLAM EGIACFAIPY T
C.eleg.UNC17  306 .......... .......... .......... .......... .......... .......... .
Drosoph.VAChT 306 DN.T-.DN.K I.MV...A.. ......VI.. ..A.KY.Q.Q .LM.AG...L ..FS..I..F C
A.gamb.VAChT  306 DNLT-.DN.K I.MV...A.. ...F..II.. ..AAQY.DKQ .LM.AG...L ..LC..I..F S
Torca         306 KT.N-ASE.Q M.IT...A.. ..I....I.. .LAAKY.NYQ .FYGA...VI I.ASSCT..A C
                                      TM8                                       TM9
O.volv.VAChT  367 TSVMQLVIPL SFVCFGIALI DTSLLPMLVH LVDTRHVSVY GSVYAIAD-- ---------- -
C.eleg.UNC17  367 .......... .......... ........G. .......... ........IS YSLAYAFGPI I
Drosoph.VAChT 367 SGYKM.ML.I CVI....... ..A...T.GY ...V.Y.... ..I.....IS YSIAYAVGPI I
A.gamb.VAChT  367 S.YIM.M..I CGI....... ..A...T.GY ...I.Y.... ..I.....IS YSLAYAVGPI I
Torca         367 RNFEE.I... CAL......V ..A...T.AF ...I.Y.... ........IS YSVAYALGPI M
                                      TM10                                      TM11
O.volv.VAChT  428 ---------- ---------- ---------- ---------- ---------- ---------- -
C.eleg.UNC17  428 AGWIVTNWGF TALNIIIFAT NVTYAPVLFL LRKVHSYDTL GAKGDTAEMT QLNSSAPAGG Y
Drosoph.VAChT 428 AGGVVEAIGF TALNFLIAFS NLAYVPVLRK LRNIYDFKPF EN-----EAN ILMQDPPNKE Y
A.gamb.VAChT  428 AGGVVEAIGF TALNFLIAFS NLLYAPVMYY LRNIYDFKHF EN-----EAN VLMGDPPTKE Y
Torca         428 AGQIVHDLGF VQLNLGMGLV NILYAPALLF LRNVCQMKPS LS-----ERN ILLEEGPKGL Y
                                  TM12
O.volv.VAChT  489 ---------- ---------- ---------- ---------- ---------- ---------- -
C.eleg.UNC17  489 NGKPEATTAE SYQGWEDQQS YQNQAQIPNH AVSFQDSR-- ---------- --------PQ A
Drosoph.VAChT 489 QTYVMHDQKP VEGGVKNHLE YGQQYQQ--- EQETNLDD-- -QQYEYQQQQ QGYQQGYQQD Q
A.gamb.VAChT  489 QTYTMHDQQM VGEEYKNHLE YGRQTDDGNY QQETNIDQGY SQNGSYEQQQ Q--QGGYQN- -
Torca         489 DTIIMEERKA AKEP------ HGSSSGN--H SVHAVLSD-- ---------- ---------- -

O.volv.VAChT  550 ---------- ---------- ---------- ---------- ---------- -----
C.eleg.UNC17  550 EFPAGYDPLN PQW------- ---------- ---------- ---------- -----
Drosoph.VAChT 550 GYQPGYQEQG GSYAPQGQPR VANPFQQQQQ QQQQQQQQVQ SRGPAAPANP FRQGF
A.gamb.VAChT  550 -YQPGYQEQG GS-------- ---VYQQQQQ QAPRHLPQQ- ---PQPVANP FRHG-
Torca         550 --QEGYSE-- ---------- ---------- ---------- ---------- -----
```

**Figure 6: Alignment of amino acids sequence of *O. volvulus* VAChT and other VAChTs**
The alignment was done with the *Caenorhabditis elegans* (unc17 CAEEL P34711), *Drosophila melanogaster* (VAT DROME 017444), *Anopheles gambiae* (Q7PR83 ENSANGP00000001263) and *Torpedo californica* (AAC59647.1). The standard single-letter abbreviations for amino acids are used. Dashes indicate gaps that have been introduced to optimize the alignment, dots indicate identity to *O. volvulus* VAChT sequence. Underlined sequences indicate the putative transmembrane domains (TM1 to TM12). Amino acid totals: *O. volv*. 406; *C. eleg*.532; *Drome*. 578; *A.gamb*. 551; *Torca.* 568. Sequences were aligned with Clustal X (1.81) computer program.

#### 4.1.3.4 Evolutionary analysis of VAChT and MATs amino acids sequences

The cloned, sequenced and aligned partial protein of *O. volvulus* VAChT was used in the construction of a neighbour joining (NJ) tree together with the corresponding partial protein portions of the already known complete VAChTs and MATs from vertebrates and invertebrates. Phylogenetic analysis of 25 homologous VAChT and VMAT sequences was undertaken using data generated from analysis of similarities and differences in the amino acids. The analysis grouped the genes/organisms into 4 major clusters/clades (1-4) (Fig. 7).

The predicted protein from this study,ONCHO VOLV. (Fig. 7) is closely related to *C. elegans* unc17 P34711 and *C. braggie* Q61MW4 (Fig. 7) and are grouped in the same cluster (Fig. 7; clade 1). Clade 1 appears quite distinct from other invertebrates' and all vertebrates' VAChTs and VMATs included in the analysis and diverged early during evolution in comparison to other organisms. Clade 2 for the insect and mollusc VAChTs, clade 3 for verterbrate VAChTs (mammals, birds and fish) and clade 4 for all VMATs of both vertebrates and invertebrates have got distinct evolutionary relationships (Fig. 7). There is much genome duplication in clades 3 and 4 as compared to clade 2 (Fig. 7). The VMATs are grouped in one clade (clade 4) indicating close genetic relationship amongst VMATs of various species whereas the VAChTs are categorized into 3 clades (clades 1-3) indicating distinct evolutionary divergence amongst VAChTs. The data generated by the neighbour joining tree indicates evolutionary relatedness amongst VMATs and evolutionary divergence amongst VAChTs. The conservation of these genes, however, amongst various animal species ranging from worms (invertebrates) to humans (vertebrates) suggests that the genes may be essential for survival.

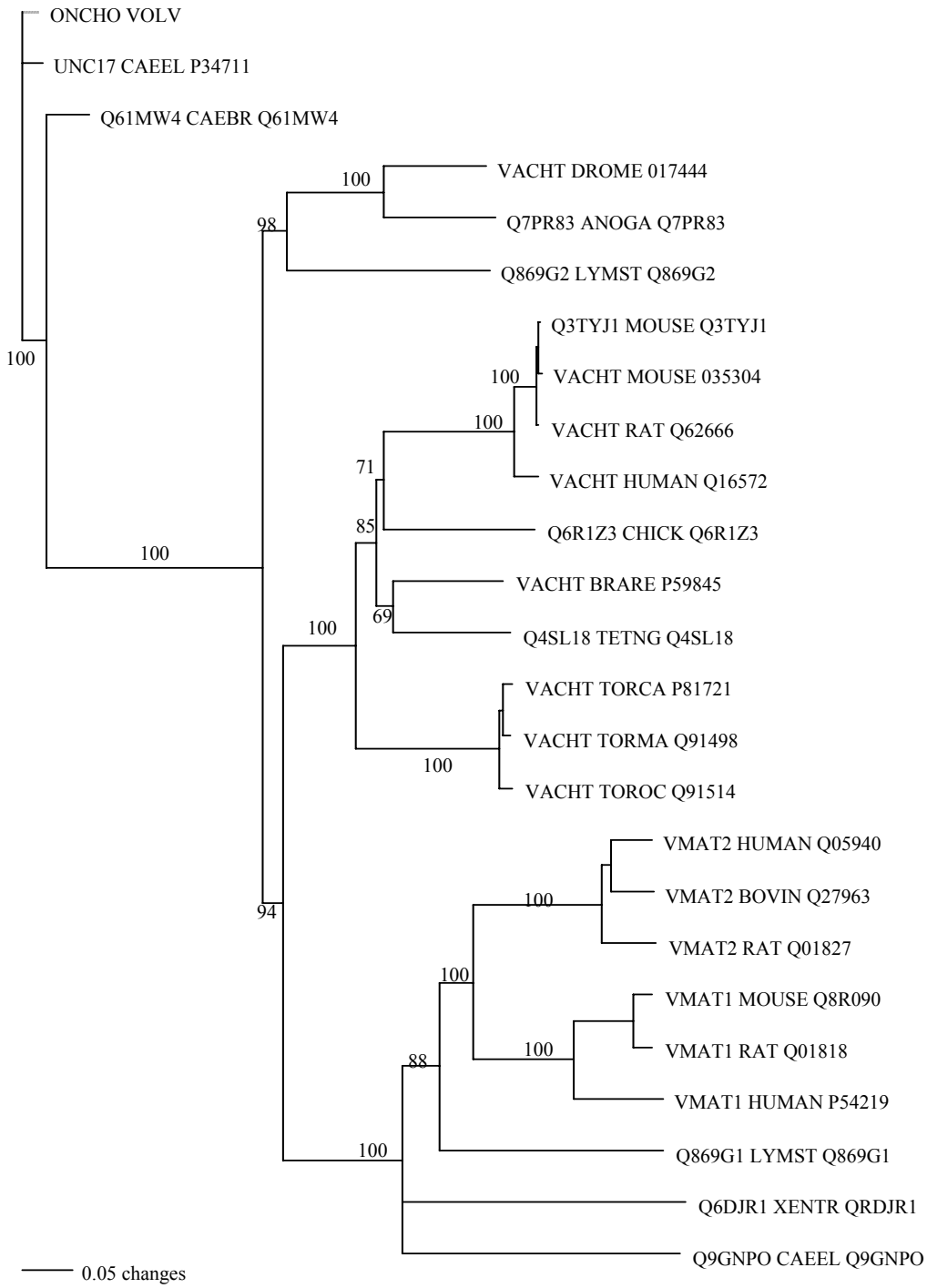


**Figure 7: Phylogenetic relationship of *O. volvulus* VAChT, other VAChTs and VMATs**
Analysis was done using amino acid sequences with the Paup 4.0b1 computer program. CAEEL, *Caenorhabditis elegans*; DROME, *Drosophila melanogaster*; ANOGA, *Anopheles gambiae*; LYMST, *Lymnea stagnalis*; CHICK, Chicken; BRARE, *Brachydanio rerio* ; TETNG,*Tetraodon nigroviridis*; TORCA, *Torpedo californica*; TORMA, *Torpedo mammillata*; TOROC, *Torpedo ocellata*; BOVIN, Bovine, XENTR, *Xenopus tropicalis*; The GenBank Accession numbers are shown on the right. Numbers at the branches indicate bootstrap proportions on NJ tree.

#### 4.1.3.5 SDS-PAGE and Western blot analysis of *O. volvulus* VAChT protein

The Coomassie Brilliant Blue stained SDS gel verified the presence of dense protein bands of variable molecular weights. The worms have few proteins in comparison to the rat protein extract (Fig. 8; Panel A). The affinity purified rat anti-vesicular acetylcholine transporter antibody detected a protein band of about 70kDa in the protein extract from the rat brain tissue but not in *O. volvulus* or *H. contortus* worm crude protein extracts (Fig. 8; Panel B).

The antibody recognizes a peptide sequence corresponding to the C-terminal of the cloned rat VAChT (amino acids 512-530). This sequence is highly conserved (>85% homology) with human VAChT (Varoqui *et al*., 1994) and highly variable in non mammalian hosts (Kitamoto *et al*., 2000). This probably led to inability to detect any protein band by Western blot analysis in *O. volvulus* and *H. contortus* (Fig. 8; Panel B; lanes 1 and 2). Corresponding antibodies could not be prepared in this study which could be specific for Onchocerca because the 3' end of gene which is highly variable still missing.

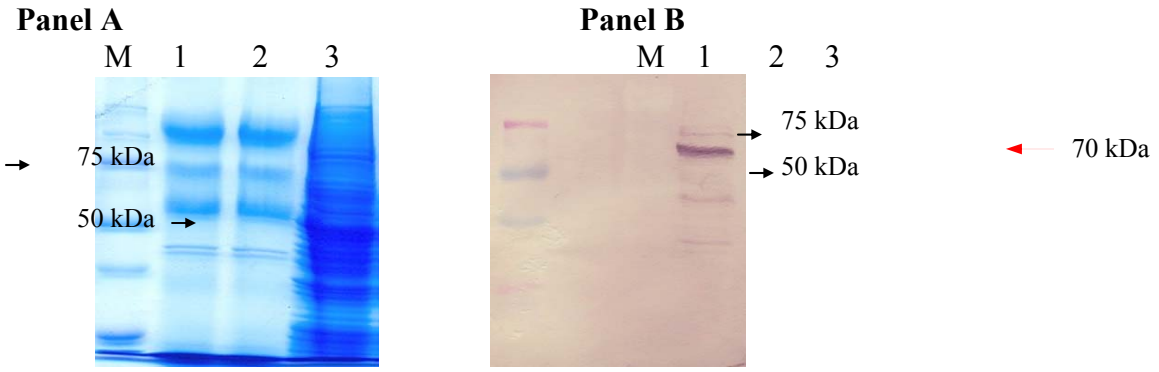


**Figure 8: SDS-PAGE and western blot results**
Total proteins recovered from cell lysates of *O. volvulus* and *H. contortus* adult female worms, and rat brain tissue were separated by SDS-10% polyacrylamide gel electrophoresis. Coomassie Brilliant Blue staining of proteins (Panel A) and corresponding Western blot analysis of total protein extracts probed with the rat anti-VAChT antibody (Panel B). Molecular weight standards, lane M, total cell lysates of *O. volvulus, H. contortus* and rat brain tissues in lanes 1, 2 and 3 respectively (Panels A and B). Protein marker sizes are indicated in kilodaltons on the left of each panel, and the VAChT protein (70 kDa) in the rat brain protein extract by the red arrowhead on the right (Panel B, lane3). Additional faint bands representing degraded and/or non-specifically bound proteins appeared in the same lane as rat VAChT. The Western blot reaction was visualized by bromochloroindolyl phosphate/nitroblue, tetrazolium solution (BCIP/NBT) (Sigma) colour reagent substrate.

## 4.2 *In vitro* evaluation of vesamicol on motility in *H. contortus*

The mean percent inhibition in motility of the *H. contortus* female adult worms caused by vesamicol increased with time and inhibitor concentration, and reached $E_{max}$ (maximum response) of 100% inhibition with 1mM vesamicol concentration after 2 hours of incubation. Lower concentrations (0.5mM or 0.1mM) of vesamicol, however, never attained 100% inhibition even at the end of the three hours of incubation (Figure 9). Fifty percent (50%) inhibition with 1mM and 0.5mM vesamicol was attained after 10 and 90 minutes respectively, while 0.1mM vesamicol never attained 50% inhibition in worm motility even at the end of the 3 hours of incubation.

Analysis of variance revealed a significant ($p<0.0001$) difference between the mean percent inhibition in motility caused by the different concentrations of vesamicol (Fig. 9; Appendix 8.4), suggesting that inhibitor concentration was a factor in increasing the mean percent inhibition in worm motility. The analysis also revealed a significant ($p=0.007$) difference between the mean percent inhibitions in motility at the different periods of incubation (Fig; 9; Appendix 8.5), suggesting that the period of incubation was also a factor increasing the mean percent inhibition in worm motility.

There was correlation between the concentration of vesamicol and the period of incubation such that increasing either the concentration of the inhibitor or the period of incubation led to an increase in the percent inhibition in worm motility.

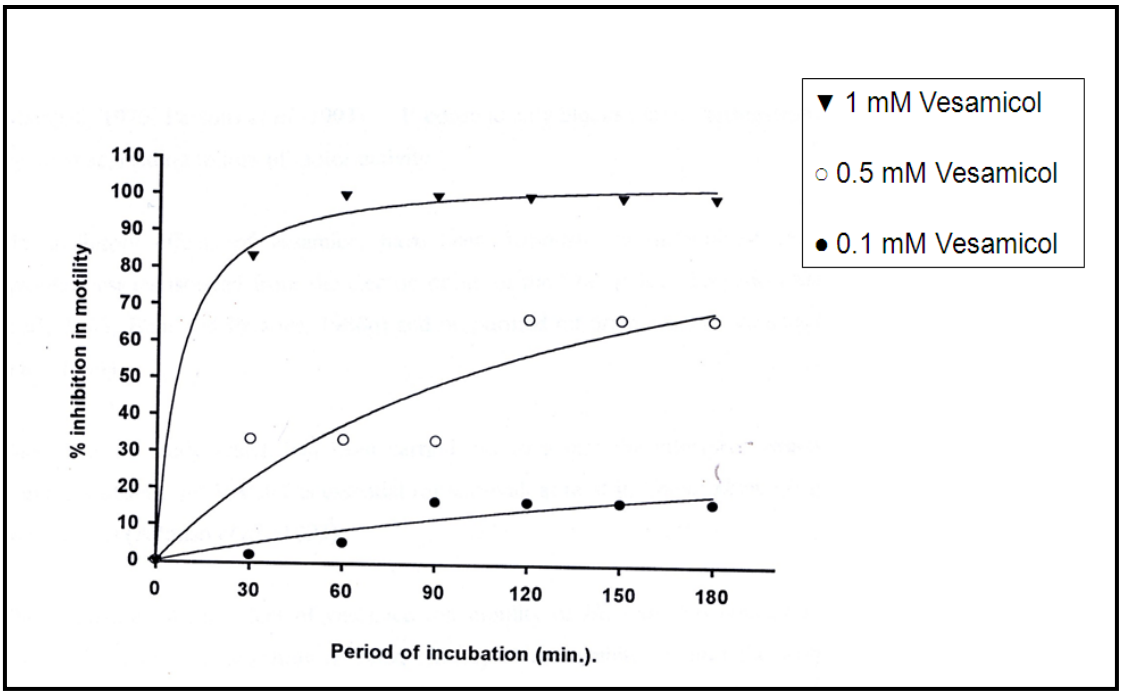


**Figure 9**: **Time-course curves showing the % inhibition in worm motility at different vesamicol concentrations**

The *H. contortus* worms were incubated at $37^0$C in physiological saline containing 0.1 mM, 0.5 mM and 1 mM vesamicol and percentage inhibition in worm motility calculated at 30 minute intervals for a period of 3 hours. Closed circles, open circles and triangles represent 0.1 mM, 0.5 mM and 1 mM vesamicol concentrations respectively. The data are from a representative experiment performed in duplicate, and the experiment was repeated twice with essentially identical results.

# CHAPTER FIVE

# DISCUSSION

## 5.1 Cloning and characterization of *O. volvulus* VAChT

### 5.1.1 Identification and verification of *O. volvulus* VAChT cDNA

The neurotransmitter acetylcholine (ACh) (Varoqui and Erickson, 1997) is synthesized in the cytosol of cholinergic nerve terminals, transported into synaptic vesicles, and secreted upon calcium influx triggered by an extracellular signal. The vesicular acetylcholine transporter (VAChT) transports this Ach from the cytoplasm into synaptic vesicles in exchange for two protons (Schuldiner, 1994; Varoqui and Erickson, 1997). The VAChT is localized to the membrane of the small synaptic vesicle in central cholinergic neurons (Gilmor *et al*., 1996) and synaptic-like microvesicles (SLMV) and endosomes in PC12 cells (Weihe *et al*., 1996; Liu and Edwards, 1997; Eiden, 1998).

Functional cholinergic neurons make and use Ach as a neurotransmitter, and must co-ordinately express choline acetyltransferase (ChAT), essential for the formation of Ach and vesicular Ach transporter (VAChT) needed for accumulation of Ach into synaptic vesicles (Rand and Russell, 1984). This work represents the first isolation and characterization of a VAChT gene from a parasitic helminth/organism.

#### 5.1.1.1 Search of dEST for VAChT homologues

The *C. elegans* unc17 and partial *O. volvulus* VAChT sequences were used to screen the NCBI data of Expressed Sequenced Tags (dEST) database containing the filarial genome EST sequences from multiple developmental stages of *O. volvulus.* Approximately 15,000 EST sequences of *O. volvulus* were screened and none showed high levels of similarity. This is suggestive of low levels of gene expression for the

various worm developmental stages. Since homologues for this gene were still missing in the genomes of Onchocerca and other filarial nematodes, further analysis to establish its chromosome location was not possible, however, human VAChT has been localized on chromosome 10 (Varoqui and Erickson, 1996).

#### 5.1.1.2 Homology of VAChT like protein from *O. volvulus*

The main line of evidence which supports the conclusion that the longest cloned PCR fragment participates in vesicular acetylcholine transport is that, it forms an open reading frame which predicts a protein of 404 amino acids which represents sequence similarities to vesicular transporter proteins (Alfonso *et al.*, 1993; Krejjei *et al*., 1993; Howell *et al.*, 1994; Roghani *et al*., 1994; Bejanin, *et al*., 1994; Erickson *et al*., 1994; Varoqui and Erickson, 1994; Erickson *et al*., 1996; Song *et al*., 1997; Kitamoto *et al*., 1998). The highest identity was found with VAChTs of *C. elegans* (98%), *D. melanogaster* (56%), *A. gambiae* (55%) and various *Torpedo species* (55%) in the 404-amino acid overlap portion.

Furthermore, the 11 putative transmembrane domains characteristic of the known VAChT and MAT cDNAs are highly conserved in the encoded partial protein (Fig. 6). In particular, aspartic acid residues in TM regions 1, 6, 10 and 11 that may be required for substrate binding and cationic amine/H+ antiport (Erickson and Eiden, 1993) are conserved.

Like the VAChTs and MATs, a large luminal loop is predicted to occur between the first two transmembrane domains (Fig. 6). The *O. volvulus* VAChT cDNA sequence for the isolated gene portion is very similar to *C. elegans* unc17. The highest divergence between the *O. volvulus* sequence and that of the *Drosophila* or *Torpedo* species is within the large hydrophilic loop and in the N-terminal end. In addition, the loop contains 2

potential sites for N-linked glycosylation in *O. volvulus* (Fig. 6). Sequence conservation of charged and polar amino acids within the putative hydrophobic transmembrane domains of VAChT suggests functional importance. Kim *et al*., (1999) showed that, Asp-398 in TM10 and Asp-425 in TM11 possess important roles in acetylcholine transport. These observations further suggest that the *O. volvulus* protein inferred in this study belongs to the family of vesicular neurotransmitter transporters/H+ antiporters characterized by 12 transmembrane domains. However, in this study, the last expected ($12^{th}$) transmembrane domain is still lacking as the 3' end was not successfully isolated. The conservation of the VAChT gene amongst evolutionary separated species indicates its functional significance. The dissection of the underlying mechanisms should reveal new and important clues to the control of eukaryotic gene expression.

The coding sequence of the mammalian, fish, mollusc and arthropod VAChT gene is not interrupted by introns (Bejanin *et al*., 1994; Kitamoto, *et al*., 1998), however, an intron is present in *C. elegans* unc17 (Kitamoto *et al.*, 1998). The *O. volvulus* VAChT genomic organization is still unknown since the 3' end is still lacking, however, the isolated portion is not interrupted by introns as revealed by PCR amplication of similar DNA fragments from both genomic and cDNA template sources. Since *C. elegans* unc17 and *O. volvulus* VAChT showed greatest degree of sequence identity (98%), it is probable that an intron may also be present in the missing gene portion for Onchocerca. Failure to obtain the 3' end from the cDNA may have been due to low levels of gene expression from the materials used for library preparations or presence of intron in genomic DNA sources.

#### 5.1.1.3 Evolutionary relationship of *O. volvulus* VAChT and other family members

The *O. volvulus* VAChT protein showed characteristics of a larger protein family

designated VAChT and MATs with homologues from mammal, bird, fish, mollusc, arthropod and worm origins. Cladograms (Fig. 7) summarizing the likely phylogeny of *O. volvulus* VAChT, grouped it together with *C. elegans* unc17 and *C. braggie* VAChT forming a mono phylogenetic group distinct from other organisms and this group emerged very early during evolution. This data is consistent with previous phylogenetic analyses based on nuclear, mitochondrial and ribosomal genes. In particular, the clustering of *O. volvulus* with two *Caenorhabditis* species into a distinct group/cluster relatively distant from other invertebrates and vertebrates supports the assumption that worms may not be closely related to other animal species. Apparently, such differences may be utilized in the development/ evaluation of potential anthelmintic drug/vaccine targets.

### 5.1.2 Immunoblot analysis and antibody specificity of VAChT proteins

The Coomassie Brilliant Blue stained SDS gel verified the presence of dense protein bands of variable molecular weights in total protein extracts of rat brain and whole worm extracts of *H. contortus* and *O. volvulus* (Fig. 8; Panel A). There were fewer protein bands in worms compared to the rat (mammal) extract which may be due to the genetic make up of the different organisms. Few protein bands have been shown in *H. contortus* adult worms (Lubega and Prichard, 1991). The affinity purified rat anti-vesicular acetylcholine transporter antibody detected a protein band of about 70kDa in the protein extract from the rat brain tissue but not in *O. volvulus* or *H. contortus* whole worm protein extracts (Fig. 8; Panel B). This antibody recognizes a peptide sequence corresponding to the C-terminal of the cloned rat VAChT (amino acids 512-530). This sequence is highly conserved (>85% homology) in human VAChT (Varoqui *et al*., 1994) and very divergent in non mammalian organisms. The protein is enriched in brain areas

known to receive dense cholinergic innervation, such as striatum, hippocampus, and amygdala, with the lowest levels in cerebellum where there is little cholinergic input (Varoqui *et al*., 1994).

The C-terminal end is the most variable region of this gene (Erickson *et al*., 1994) and therefore rat anti-VAChT antibodies were probably very specific to the point of not detecting the protein from non mammalian organisms of *O. volvulus* and *H. contortus* by Western blot analysis. Nevertheless, the analysis enabled direct characterization of the encoded protein in rat brain tissue and confirmed the specificity of antibodies designed from the variable C- terminal end of the VAChT protein.

Due to the absence of the complete protein and failure to produce specific antibodies from the variable 3' end region of the *O. volvulus* VAChT protein sequence, the anticipated corresponding protein could not be detected in worm tissue protein extracts. Basing on the cDNA sequence of *C. elegans* unc17 (Alfonso *et al*., 1993), however, a protein of approximately 55kDa was expected. Interestingly, although *O. volvulus* is very similar to *C. elegans* for the VAChT gene, it typically leads a parasitic life while enclosed in nodules compared to *C. elegans* which is a free living nematode. The readily available free living nematodes can be used/ exploited for chemotherapeutic approaches, such as, models to study the anticipated possible drug targets for parasitic nematodes especially for proteins/pathways showed by the two nematode worms. A possible impact for the development and evaluation of an effective treatment for human onchocerciasis and other filariases could be evaluated in respect to the importance of the VAChT gene for survival.

### 5.1.3 Anthelminthic drug/vaccine target potential of VAChTs

Transmembrane transport processes are fundamental for all living organisms. This

is in particular reflected by the fact that close to 300 transport proteins are encoded by the human genome (Stevens and Arkin, 2000). Many of these are expressed in the brain and known to play a pathophysiological role in the development of mental disorders as well as neurological and neurodegenerative diseases. In light of the critical physiological function of neurotransmitter transporters it is not surprising that several of these are important drug targets. Examples include the transporters for serotonin, dopamine and norepinephrine that are targets for the action of antidepressants and widely abused psychoactive compounds such as cocaine, amphetamine and transporters for GABA as targets for antiepileptic drugs. However, still many transport proteins in the brain represent unexplored targets for the action of putative new drugs.

Vesicular acetylcholine transport is essential in cholinergic neurotransmission because homozygous VAChT knockout mutants in *C. elegans* (Alfonso *et al*., 1993) and *D. melanogaster* (Kitamoto *et al*., 1998) do not live for more than a few days. Vesamicol ((-)-trans-2-(4-phenylpiperidinol) cyclohexanol) has been shown to be a specific inhibitor of Ach by binding on VAChT receptors and it acts in a noncompetitive manner (Bahr *et al*., 1992; Parsons *et al*., 1993).

To address the potential role of the identified *O. volvulus* VAChT in drug and/or vaccine development against onchocerciasis and other filariases, recombinant protein will be prepared so that specific anti-sera could be raised and used to precisely define the localization of this protein in the worms. This was one of the aims of this study which was not achieved due to the failure to isolate the 3' end of the gene which would have been used to produce the specific antibodies. It may also be possible that the comparison of the inhibition characteristics of the recombinant worm and human VAChT proteins may reveal compounds which may specifically inhibit the worm protein. Alternatively,

non-cross reactive antibodies may be produced and used in tackling the parasites.

### 5.2 Effect of vesamicol on motility in *H. contortus* adult worms

Vesamicol is a potent non-competitive inhibitor of the vesicular acetylcholine transporter (Marshall, 1970; Parsons *et al,* 1993). It consequently blocks nerve transmission across the synapse, leading to loss of motor activity. The inhibitory effects of vesamicol have been demonstrated on purified cholinergic synaptic vesicles isolated from the electric organ of the Marine Ray Torpedo (Anderson *et al.,* 1983*;* Bahr and Parsons, 1986b) and on purified rat brain synaptic vesicles (Haigh *et al*., 1994).

There was no study which had been carried out to assess the effects of vesamicol in viable organisms, yet VAChT is essential for survival, at least in *Caenorrhabditis elegans* adult worms (Alfonso *et al*., 1993). The evaluation of the effect of vesamicol on motility of *Haemonchus contortus* female adult worms done in this study revealed efficacy of the inhibitor against the worms. As revealed from the time-response graph (Fig. 9), the percent inhibition of worm motility increased significantly ($p<0.0001$) with increase in the concentration of vesamicol.

The increase in percent inhibition of worm motility with increasing concentration of the inhibitor was probably due to the progressive saturation of the receptor sites on the vesicular acetylcholine transporter (VAChT), as the inhibitor concentration increased up to about 1mM at which all the receptor sites on the VAChT were saturated by the inhibitor, hence suppressing the activity of acetylcholine, leading to paralysis and immotility.

The percent inhibition in worm motility increased significantly ($p<0.007$) with increasing period of incubation (Fig. 9). This suggests that the amount of inhibitor bound to the receptor sites of the VAChT increased with increase in the period of

incubation. This is in accordance with Lullmann *et al*., (1993), who observed that the amount of a compound absorbed and distributed in the body increased with time.

A hundred percent (100%) inhibition was not attained with concentrations of vesamicol lower than 1mM even at the end of the 3 hour incubation period, probably because at lower concentrations and earlier periods of incubation, the threshold concentration of vesamicol had not been reached for vesamicol to displace all the acetylcholine from the receptor sites on the VAChT protein.

The thick nematode cuticle of *Haemonchus contortus* (Soulsby, 1982) could have added as a barrier to penetration of the inhibitor, since such barriers reduce permeability of compounds into organisms (Lullmann *et al.,* 1993). This suggests that only vesamicol dilutions of about 1mM raise enough molecules (threshold concentration), to saturate the receptor sites on the VAChT protein.

Dilutions of vesamicol at 1mM caused a maximum effect (Emax) of 100% inhibition of motility so that a further increase in the concentration of the inhibitor could not cause any further increase in percentage inhibition of worm motility. This suggests that all the receptor sites on the VAChT had been saturated at 1mM vesamicol. The observation that the time taken to attain 50% inhibition was shorter with higher (1mM) concentrations than with lower (0.5mM) concentrations of vesamicol, may be urged that the amount of compound absorbed and distributed in the body increases with time (Lullman, *et al*., 1993), so that more inhibitor molecules were required to cause the same response (50% inhibition) at an earlier time than at a later time. The results of data analysis revealed that inhibition is attained either by increasing the concentration of vesamicol or/and exposure time.

# CHAPTER SIX

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusions

(i) The *Onchocerca volvulus* VAChT identified in this study plays a role in cholinergic neural transmission. This is supported by the fact that its homologues, unc17 in *C. elegans*, its mutations protect against organophosphorus toxicity and the Torpedo electric lobe provides extremely dense cholinergic innervation to the electric organ. Uncoupling/inhibition of VAChT gene expression in *O. volvulus* may lead to a reduction in vesicular ACh pools resulting in presynaptic cholinergic hypofunction which may be a lethal phenotype.

(ii) The vesamicol VAChT inhibitor is effective against the *Haemonchus contortus* female adult worms by loss of motility. The efficacy of the inhibitor against the worms is dependent on the period of exposure and the concentration of the inhibitor to which the worms are exposed.

## 5.2 Recommendations

(i) The possibility that, like vesicular monoamine transport (Liu *et al*., 1992) and unc 17 vesicular Ach transport (Alfonso *et al*., 1993) protect against certain forms of neural toxicity due to either endogenous or exogenous compounds could be explored in *Ov*VAChT.

(ii) To address the potential role in disease and in development of drug and /or vaccine targets, recombinant *O. volvulus* VAChT protein could be prepared so that antisera can be raised and then used to more precisely define the localization and biochemical effects of this protein within the worm.

(iii) The inhibition characteristics of the recombinant *O. volvulus* and recombinant

human VAChT proteins may be compared to reveal a compound(s) which can specifically inhibit the parasite protein or non cross reactive antibodies may be produced which may be useful in tackling the parasite's defense.

(iv) Point mutations may be induced in the conserved and non conserved nucleotides within the *Ov*VAChT gene when the complete gene has been isolated to determine which amino acids are essential for substrate transport.

(v) Investigations such as selective toxicity of vesamicol analogues in ruminants would be of paramount importance, as they would help to establish the effective doses for treatment of nematodiasis *in vivo* without endangering the life of the host.

(vi) The vesamicol and its analogous may also be assessed on other nematodes to evaluate whether they have a broad-spectrum effect on nematodes. This would also yield good preliminary results towards development of anthelmintics that can act on the nervous system.

(vii) Other analogues of vesamicol such as L-(+) vesamicol hydrochloride may also be assessed for their effects on the *H. contortus* worms.

(viii) The effects of vesamicol on other parameters of worms' viability such as, egg hatchability, growth and larval development may also be considered to ascertain the effect of the inhibitor on the worms.

## CHAPTER SEVEN

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# CHAPTER EIGHT

# APPENDICES

## 8.1 Solutions for Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot assays.

(i) **30% acrylamide/bis acrylamide**

87.6g Acrylamide

2.4g N'N'-bis-methylene-acrylamide

300ml distilled water

(ii ) **10% APS (Ammonium persulphate)**

0.1g Ammonium persulphate

1ml distilled water

(iii) **1.5M Tris-HCl, pH 8.8**

27.23g Tris base

150ml distilled water

Adjust pH with 6N HCl

(iv) **0.5M Tris-HCl, pH 6.8**

6g Tris base

100ml distilled water

Adjust pH with 6N HCl

(v) **10% SDS (Sodium dodecyl sulphate)**

10g SDS

100ml distilled water

(vi) **Separating gel buffer**

75ml 1.5M Tris-HCl, pH 8.8

4ml 10% SDS

21ml distilled water

(vii) **Stacking gel buffer**

50ml 0.5M Tris-HCl, pH 6.8

4ml 10% SDS

46ml distilled water

(viii) **1% Bromophenol blue**

1g Bromophenol blue

100ml distilled water

(ix) **SDS sample reducing buffer**

3.8ml distilled water

1ml 0.5M Tris-HCl, pH 6.8

0.8ml glycerol

1.6ml 10% SDS

0.4ml 2-mercaptoethanol

0.4ml 1% bromophenol blue

(x) **10% Separating gel solution**

4ml 30% acrylamide/bis acrylamide

3ml separating gel buffer

4.94ml distilled water

60µl 10% APS

10µl TEMED (Tetra ethyl methyl ethylene diamine)

(xi) **4% stacking gel solution**

0.64ml 30% acrylamide/bis acrylamide

1.2ml stacking gel buffer

2.935ml distilled water

24µl 10% APS

5µl TEMED

(xii) **5X Electrode buffer**

3g Tris base

14.4g glycine

1g SDS

1000ml distilled water

(xiii) **Coomassie Blue R-250 staining buffer**

0.5g Coomassie R-250

225 absolute methanol

225 distilled water

50ml acetic acid

(xiv) **Destaining buffer**

100ml absolute ethanol

100ml acetic acid

800ml distilled water

(xv) **1X Transfer buffer**

9g glycine

1.93g Tris base

1000ml distilled water

(xvi) **1X PBS (Phosphate buffered saline)**

8g Sodium chloride

0.2g Potassium chloride

1.44g Disodium hydrogen phosphate

0.24g Potassium dihydrogen phosphate

1000ml distilled water

(xvii) **3% BSA (Bovine serum albumin)**

3g BSA

100ml 1X PBS

(xviii) **1X PBS/TWEEN-20**

500ml 1X PBS

250µl TWEEN-20

**8.2 Buffer for incubation of *H. contortus* worms *in vitro***

**(i) Physiological saline (0.8%)**

8g NaCl

1.5g $Na_2HCO_3$

1000ml distilled Water

Sterilized by autoclaving and used or stored at $37^oC$

**8.3 Buffers for agarose gel electrophoresis**

(i) **50X TAE (Tris acetate ethylenediaminetetra-acetic acid)**
242g Tris base
57.1ml Glacial acetic acid
100ml $Na_2$ EDTA pH 8.0

(ii) **1X TAE (40mM Tris acetate buffer, pH 8.0, 1mM $Na_2$ EDTA)**
40ml 50X TAE buffer
1960ml distilled water

(iii) **1% agarose**
0.3g agarose
30ml 1X TAE buffer
Heated in autoclave and cooled to $55^0C$

**8.4 The mean percent inhibition in motility of *Haemonchus contortus* adult worms by various vesamicol concentrations and time periods**

| Concentration of Vesamicol (µM) | Mean % inhibition of motility | | |
|---|---|---|---|
| | After 1 hr | After 2 hrs | After 3 hrs |
| 0 | 0 | 0 | 22.2 |
| 100 | 5.2 | 16.7 | 16.7 |
| 300 | 16.7 | 33.3 | 50.0 |
| 500 | 41.7 | 58.3 | 66.7 |
| 700 | 50.0 | 66.7 | 100 |
| 900 | 83.3 | 100 | 100 |
| 1000 | 100 | 100 | 100 |

**8.5 The mean percent inhibition in motility of *Haemonchus contortus* adult worms by various vesamicol concentrations**

| Period of incubation (Minutes) | Mean % Inhibition of Motility | | |
|---|---|---|---|
| | 0.1mM Vesamicol | 0.5mM Vesamicol | 1mM Vesamicol |
| 0 | 0.0 | 0.0 | 0.0 |
| 30 | 1.5 | 33.3 | 83.3 |
| 60 | 5.2 | 41.7 | 100 |
| 90 | 16.7 | 50.0 | 100 |
| 120 | 16.7 | 58.3 | 100 |
| 150 | 16.7 | 66.7 | 100 |
| 180 | 22.2 | 66.7 | 100 |

## 8.5 An output of the blast analysis of the partial O. *volvulus* VAChT gene at NCBI

| Alignment | DB:ID | Source | Length | Score | Identity% | Positives% | E() |
|---|---|---|---|---|---|---|---|
| 1 | UNIPROT:UN17_CAEEL | Vesicular acetylcholine transporter unc-17 (Uncoordinated protein 17). | 532 | 2068 | 98 | 98 | 0.0 |
| 2 | UNIPROT:Q61MW4 | Hypothetical protein CBG08307. | 530 | 1992 | 94 | 97 | 0.0 |
| 3 | UNIPROT:VAT_DROME | Vesicular acetylcholine transporter (VAChT). | 578 | 1159 | 56 | 71 | e-114 |
| 4 | UNIPROT:Q7PR83 | ENSANGP00000001263 (Fragment). | 551 | 1096 | 54 | 70 | e-107 |
| 5 | UNIPROT:Q869G2 | Vesicular acetylcholine transporter. | 585 | 1066 | 50 | 65 | e-104 |
| 6 | UNIPROT:VAT_TORCA | Vesicular acetylcholine transporter (VAChT) (TorVAChT). | 515 | 1044 | 55 | 70 | e-102 |
| 7 | UNIPROT:VAT_TOROC | Vesicular acetylcholine transporter (VAChT) (Vesamicol binding protein). | 511 | 1043 | 55 | 70 | e-101 |
| 8 | UNIPROT:VAT_TORMA | Vesicular acetylcholine transporter (VAChT) (Vesamicol binding protein). | 511 | 1043 | 55 | 70 | e-101 |
| 9 | UNIPROT:VAT_BRARE | Probable vesicular acetylcholine transporter (VAChT). | 493 | 984 | 52 | 66 | 2e-95 |
| 10 | UNIPROT:VAT_RAT | Vesicular acetylcholine transporter (VAChT) (Solute carrier family 18 member 3) (rVAT). | 530 | 955 | 49 | 64 | 2e-92 |
| 11 | UNIPROT:VAT_MOUSE | Vesicular acetylcholine transporter (VAChT) (Solute carrier family 18 member 3). | 530 | 951 | 49 | 64 | 6e-92 |
| 12 | UNIPROT:Q6R1Z3 | Vesicular acetylcholine transporter. | 522 | 933 | 50 | 63 | 5e-90 |
| 13 | UNIPROT:VAT_HUMAN | Vesicular acetylcholine transporter (VAChT) (Solute carrier family 18 member 3). | 532 | 932 | 49 | 63 | 6e-90 |
| 14 | UNIPROT:Q8STE3 | Vesicular acetylcholine transporter. | 657 | 901 | 48 | 62 | 1e-86 |
| 15 | UNIPROT:VMT2_MOUSE | Synaptic vesicular amine transporter (Monoamine transporter) (Vesicular amine transporter 2) (VAT2) (Solute carrier family 18 member 2). | 517 | 725 | 37 | 53 | 4e-68 |
| 16 | UNIPROT:VMT2_HUMAN | Synaptic vesicular amine transporter (Monoamine transporter) (Vesicular amine transporter 2) (VAT2) (Solute carrier family 18 member 2). | 514 | 724 | 37 | 54 | 6e-68 |
| 17 | UNIPROT:VMT2_RAT | Synaptic vesicular amine transporter (Monoamine transporter) (Vesicular amine transporter 2) (VAT2) (Solute carrier family 18 member 2). | 515 | 723 | 37 | 53 | 7e-68 |
| 18 | UNIPROT:VMT2_BOVIN | Synaptic vesicular amine | 517 | 710 | 38 | 54 | 2e- |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | transporter (Monoamine transporter) (Vesicular amine transporter 2) (VAT2) (Solute carrier family 18 member 2). | | | | | 66 |
| 19 | UNIPROT:VMT1_MOUSE | Chromaffin granule amine transporter (Vesicular amine transporter 1) (VAT1) (Solute carrier family 18 member 1). | 521 | 704 | 37 | 55 | 7e-66 |
| 20 | UNIPROT:Q86NW1 | GH16917p (RH74704p). | 610 | 701 | 39 | 54 | 1e-65 |
| 21 | UNIPROT:Q8IH57 | GH10249p. | 646 | 701 | 39 | 54 | 1e-65 |
| 22 | UNIPROT:Q8IGS0 | RE38567p. | 610 | 701 | 39 | 54 | 1e-65 |
| 23 | UNIPROT:VMT1_HUMAN | Chromaffin granule amine transporter (Vesicular amine transporter 1) (VAT1) (Solute carrier family 18 member 1). | 525 | 679 | 35 | 54 | 3e-63 |
| 24 | UNIPROT:VMT1_RAT | Chromaffin granule amine transporter (Vesicular amine transporter 1) (VAT1) (Solute carrier family 18 member 1). | 521 | 676 | 36 | 53 | 6e-63 |
| 25 | UNIPROT:Q869G1 | Vesicular monoamine transporter. | 532 | 658 | 35 | 51 | 5e-61 |
| 26 | UNIPROT:Q9V6R0 | CG6119-PA, isoform A. | 498 | 621 | 37 | 53 | 4e-57 |
| 27 | UNIPROT:Q8ML63 | CG6119-PB, isoform B. | 462 | 621 | 37 | 53 | 4e-57 |
| 28 | UNIPROT:Q7QA16 | ENSANGP00000016901 (Fragment). | 453 | 613 | 35 | 53 | 3e-56 |
| 29 | UNIPROT:Q8AVC6 | MGC52635 protein. | 471 | 587 | 32 | 51 | 2e-53 |
| 30 | UNIPROT:Q9BRE4 | SLC18A1 protein. | 493 | 586 | 33 | 50 | 2e-53 |
| 31 | UNIPROT:Q9GNP0 | Abnormal catecholamine distribution protein 1, isoform a. | 553 | 564 | 39 | 58 | 4e-51 |
| 32 | UNIPROT:Q96GL6 | SLC18A1 protein. | 385 | 561 | 33 | 52 | 9e-51 |
| 33 | UNIPROT:Q618B6 | Hypothetical protein CBG14734. | 582 | 560 | 37 | 55 | 1e-50 |
| 34 | UNIPROT:Q7Z1Q0 | Abnormal catecholamine distribution protein 1, isoform b. | 557 | 520 | 34 | 50 | 2e-46 |
| 35 | UNIPROT:Q6DJR1 | Solute carrier family 18 (Vesicular monoamine), member 2. | 484 | 518 | 30 | 48 | 3e-46 |
| 36 | UNIPROT:Q6PCF0 | MGC69173 protein. | 475 | 502 | 30 | 49 | 1e-44 |
| 37 | UNIPROT:Q66L40 | Slc18a2 protein. | 447 | 452 | 39 | 53 | 3e-39 |
| 38 | UNIPROT:Q8MT20 | RH14816p. | 281 | 451 | 38 | 55 | 4e-39 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 39 | UNIPROT:Q9VCJ5 | CG10251-PA. | 558 | 375 | 30 | 47 | 4e-31 |
| 40 | UNIPROT:Q8CGL9 | Vesicular monoamine transporter 1 variant (Fragment). | 207 | 364 | 36 | 56 | 5e-30 |
| 41 | UNIPROT:Q8BKW3 | Mus musculus 7 days embryo whole body cDNA, RIKEN full-length enriched library, clone:C430018J19 product:CHROMAFFIN GRANULE AMINE TRANSPORTER (VESICULAR AMINE TRANSPORTER 1) (VAT1) homolog. | 187 | 225 | 35 | 50 | 3e-15 |
| 42 | UNIPROT:O74852 | SPCC18.02 protein. | 448 | 208 | 27 | 44 | 2e-13 |
| 43 | UNIPROT:Q99870 | Vesicle monoamine transporter type 2 (Fragment). | 160 | 207 | 52 | 68 | 2e-13 |
| 44 | UNIPROT:BMR2_BACSU | Multidrug resistance protein 2 (Multidrug-efflux transporter 2). | 400 | 191 | 22 | 40 | 1e-11 |
| 45 | UNIPROT:Q81HF9 | Multidrug resistance protein B. | 400 | 187 | 23 | 41 | 3e-11 |
| 46 | UNIPROT:Q6HMY3 | Multidrug resistance protein B. | 400 | 183 | 23 | 40 | 7e-11 |
| 47 | UNIPROT:Q703J0 | NorA protein (Quinolone resistance protein). | 387 | 181 | 25 | 50 | 1e-10 |
| 48 | UNIPROT:Q81UM9 | Multidrug resistance protein. | 400 | 178 | 22 | 39 | 2e-10 |
| 49 | UNIPROT:Q73CY6 | Multidrug resistance protein. | 400 | 177 | 22 | 40 | 3e-10 |
| 50 | UNIPROT:Q63FH7 | Multidrug resistance protein B. | 400 | 177 | 22 | 40 | 3e-10 |