Investigation into the presence of orthologues of the putative cyclooctadepsipeptide receptor HC110-R in *Toxocara canis* and *Ancylostoma caninum*

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Abstract

Parasitic nematodes cause major problems in livestock animals and companion animals. The nematode infections can also afflict human as a zoonosis.
Anthelmintic drugs are used to control, prevent and treat nematode parasite infections in both, humans and animals. Since anthelmintic drugs have been used continuously for parasite control, resistance has gradually developed and is now found in a number of parasite species against most of the anthelmintics.

The PF1022A is a cyclooctadepsipeptide isolated from the fungus *Mycelia (M.) sterilia* and it has been shown to act as a broad spectrum anthelmintic drug against a wide variety of nematodes in companion and livestock animals. From the present knowledge about the mode of action of this new class of cyclooctadepsipeptides it becomes clear that they have another mode of action than other common anthelmintics. Recently, a novel heptahelical transmembrane receptor (HC110-R) was isolated from the parasitic sheep nematode *Haemonchus contortus* as a possible target of the PF1022A. The original function of this receptor in nematodes still remains unknown.
For the understanding of the mode of action of PF1022A and clarification of the original function of this receptor it is of great importance to identify and characterise HC110-R receptor orthologues in other main target nematodes.

The aim of the present study was describe a putative HC110-R receptor orthologues in *T. canis* and *A. caninum*.

Molecular techniques as for example Southern Blot, PCR and screening of cDNA libraries have been shown to be a useful tool for the identification of those receptors (Saeger, 2001).
In the present study a genomic DNA library of *T. canis* have been constructed, a cDNA library of *A. caninum* was already present. The construction of the probes for the screening of the libraries and the Southern Blot was based on the cDNA of *H. contortus*. By Southern Blot analysis it was

possible to show putative orthologues for both parasites. However, the screening of the DNA libraries did not reveal any positive result. By PCR a short part of the sequence of *T. canis* was amplified, which showed a high similarity to the HC110-R receptor of *H. contortus* and therefore possibly belongs to the relevant orthologue. Even with a high number of performed PCR's (degenerated primer and RACE) further parts of the sequence could not be detected.

The results of this study indicate that for the further screening of libraries of *T. canis* and *A. caninum* probes have to be optimised in order to be more specific for the targeted species. The described short sequence for *T. canis* is a useful tool for the design of new primers to find the remaining parts of this putative receptor orthologues.

Keywords: *T. canis*, cyclooctadepsipeptides, HC110-R receptor

Zusammenfassung

Infektionen mit Nematoden sind eines der größten Gesundheitsprobleme in Nutztieren und Haustieren. Nematodeninfektionen können zu Zoonosen beim Menschen führen.
Anthelminthika werden zur Kontrolle, Prävention und Behandlung von Infektionen mit Nematoden sowohl beim Menschen als auch beim Tier eingesetzt. Seit dem häufigen und regelmäßigen Einsatz der Anthelminthika zur Kontrolle dieser Parasiten hat die Resistenzentwicklung stetig zugenommen, so daß heute eine große Anzahl der Nematodenarten gegen etliche der gängigen Anthelminthika resistent sind.

PF1022A gehört zu der Klasse der Cyclooctadesipeptide und wurde aus dem Pilz *Mycelia sterilia* isoliert. Es wirkt als Breispektrum-Anthelminthikum gegen eine Vielzahl von Nematoden bei Mensch und Tier. Mit dem bisherigen Wissensstand über den Wirkungsmechanismus dieses Anthelminthikums ist klar geworden, daß diese neue Wirkstoffgruppe einen anderen Wirkungsmechanismus als alle bisher bekannten Anthelminthika hat. Vor kurzem ist ein neuer heptahelikaler Transmembran-Rezeptor (HC110-R) von dem Schafparasiten *Haemonchus contortus* als ein möglicher Zielrezeptor für PF1022A isoliert worden. Bisher konnte seine natürliche Funktion im Organismus der Nematoden nicht genau geklärt. Für das genaue Verständnis des Wirkungsmechanismus von PF1022A und zur Klärung der eigentlichen biologischen Funktion des Rezeptors ist es von großer Bedeutung, einem möglichen HC110-R Rezeptor orthologen in anderen wichtigen Nematoden zu suchen.

Das Ziel der vorliegenden Arbeit war, mögliche Orthologe des HC110-R Rezeptors von *H. contortus* in den Spezies *T. canis* und *A. caninum* zu identifizieren und zu charakterisieren.
Molekulare Techniken wie zum Beispiel Southern Blot, PCR und Screening von cDNA-Banken haben sich als hilfreiche Mittel zur Identifikation solcher Rezeptoren erwiesen.

In der vorliegenden Studie wurde eine genomische DNA-Bank von *T. canis* konstruiert, eine cDNA-Bank von *A. caninum* lag vor. Die Sonden für das Screening der Banken und für die Southern Blot untersuchen wurden auf der Grundlage der Sequenz des HC110-R Rezeptors von *H. contortus* cDNA hergestellt. Im Southern Blot ließ sich für *T. canis* und *A. caninum* Orthologen zu dem *H. contortus* Rezeptor nachweisen. Das Screening der Banken zeigte jedoch kein positives Ergebnis. Mit Hilfe der PCR konnte eine Teilsequenz von *T. canis* amplifiziert werden, welche eine deutliche Ähnlichkeit zum HC110-R Rezeptor von *H. contortus* aufweist und somit vermutlich zu dem gesuchten Ortholog gehört.
Weitere Teilsequenzen konnten trotz zahlreich durchgeführter PCR's (degenerierte Primer und RACE) nicht amplifiziert werden.

Die Ergebnisse dieser Arbeit legen nahe, daß für das neuerliche Screening der DNA-banken für *T. canis* und *A. caninum* die Sonden optimiert werden müssen. Die hier beschriebene kurze Sequenz von *T. canis* dient dabei als hilfreiches Mittel um neue, spezifischere Primer herzustellen und somit eventuell den Rest des gesuchten Rezeptors finden und lokalisieren zu können.

Schlagwörter: *T. canis*, Cyclooctadepsipetide, HC110-R Rezeptor

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Abbreviations

A	Adenosine
AP	Adapter primer solution
AR	Anthelmintic resistance
ATP	Adenosin Triphosphate
bp	base pairs
BZ	Benzimidazol
C	Cytosine
cDNA	complementary DNA
CLM	Cutaneus larva migrans
cm	centimeter
CSPD	Alkaline phosphatase substrate
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTPs	Deoxinucleotide triphosphates
e.g.	example given
et al.	Et alii (and others)
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ELISA TES	Enzyme linked immunosorbent assay – *T. canis* excretory secretory
g	gram
G	Guanosine
GABA	Gamma amino butyric acid
GPCR	g-protein-coupled receptor
GSP	Gen specific primer
HC110-R	Heptahelical transmembrane receptor

h	hour
IPTG	Isopropyl β-D Thiogalactopyranoside
ITS	Internal transcribed spacers
IVM	Ivermectin
kDa	kilo Daltons
kg	kilogram
L3	third stage larvae
L4	fourth stage larvae
LB	Luria Bertani
LTX	latrotoxin
ml	mililiter
M	molar
MDR	multiple drug resistance
min.	minute
ML	macrocyclic lactones
MOPS	Morpholineopropanesulfonic acid
mRNA	messenger RNA
mtDNA	mitochondrial DNA
µ	micro
n	nano
nt	Nucleotide
OD	Optical Density
OLM	Ocular larva migrans
p	pico
P	Pyrantel
PCR	Polymerase chain reaction
PCR-RFCP	PCR-based restriction fragment length polymorphism
pfu	Plaque forming units
pi	post infection

PM	Phenothiazine
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Rounds per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
T	Thymidine
TAE	Tris-acetate buffer
Taq	Thermus aquaticus
TCBZ.SO	Triclabendazole sulfoxide
Tm	Mealting Temperature
Tris	Tris (hydroxymethyl) aminoethan
U	Unit
U/µl	Unit per microliter
UV	Ultraviolet
V/cm	Volts per centimeter
v/v	Volumen/Volumen
VLM	Viceral larva migrans
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-Galactosidase

1. Literature review

Nematode parasites such as *Toxocara canis* and *Ancylostoma caninum* cause serious problems in humans and companion animals (e.g. dogs and cats).
The new drug class of cyclooctadepsipeptides has been introduced in 1992. Resistance to some of the common anthelmintics has been reported previously for *Toxocara* spp. and *Ancylostoma* spp. in humans but so far not for *A. caninum* in dogs or *T. canis/cati* in dogs and cats. For better understanding of the mode of action of this anthelmintic drug and further development of new drugs against these diseases it is of major importance to localise and characterise the receptor for the cyclooctadepsipeptides.

1.1. *Toxocara canis*

1.1.1. General characteristics of *T. canis*

The nematode *T. canis* belongs to the superfamily Ascaridiodea within the family Toxocaridae. These roundworms of dogs are zoonotic parasites which cause also widespread and common infections in humans. Toxocarosis is the clinical term applied to infection with these nematodes. The host for *T. canis* is the domestic dog where the adult worms live in the lumen of the small intestine (Despommier, 2003; Rommel *et al.,* 2000; Glickman and Shantz, 1981). The worms have three large lips and a glandular esophageal bulb, located at the junction of the esophagus and intestine (Bowman *et al.*, 2003).

The male adult is 10-12 cm long and characterised by a curled tail (Fig. 1.1.). The female is 12-18 cm long and, in contrast to the male form, exhibits a straight tail (Despommier, 2003; Rommel *et al.,* 2000).

1.1.2. Life cycle of *T. canis*

The female worms can produce up to 200,000 eggs per day. Eggs passed out in the feces are not infective and require an incubation period of about 4 days at 30ºC in the soil to embryonate (Glickman and Shantz, 1981; Sinhg, 2003). The eggs have pitted surfaces (Bowman *et al.,* 2003), and therefore are extremely resistant to chemical and climatic agents (Levine, 1980). They can survive in an appropriate external environment up to 6 years (Mueller, 1953).

Infections can occur if the definitive host ingests viable embryonated eggs from contaminated sources as for example the soil and ingestion of infected paratenic hosts (Rommel *et al.,* 2000). Furthermore the host can get the infection *in utero* from the infected mother via intrauterine infection (Despommier, 2003) or lactogenic infection (Rommel *et al.,* 2000).

Infective larvae hatch after ingestion of eggs and penetrate through the wall of the small intestine. The further migration throughout the body can happen for month or up to several years, causing damage to the tissues they enter. There are two main ways of migration, the tracheal and the somatic migration.

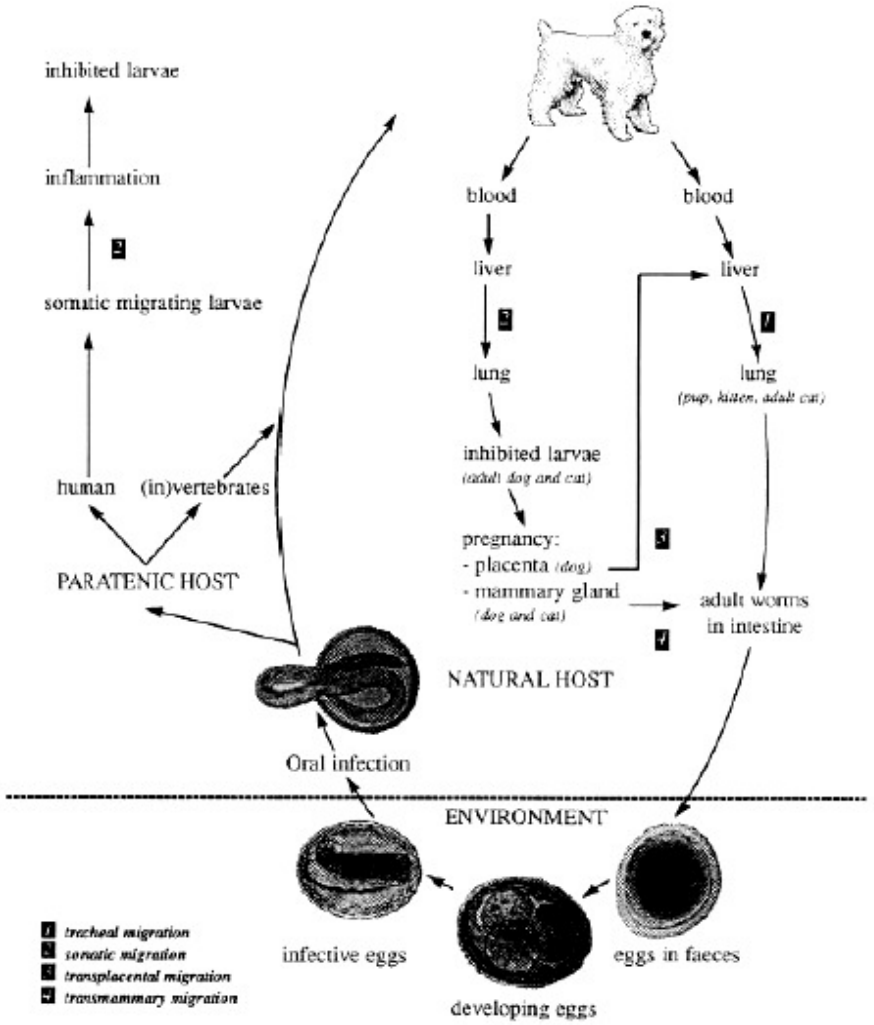
In the tracheal migration the infective L3 wander with the bloodstream into the liver and the lungs and from there via trachea and esophagus back into the small intestine where they finally reach maturity. The larvae can be observed in the liver one or two days post-infection (pi) and in lung, trachea, pharynx and small intestine 10 days pi (Rommel *et al.,* 2000).

In the somatic migration the larvae wander with the bloodstream into the lungs, where they penetrate through capillary vessels into the systemic circulation system. With the blood they reach their different destination organs where they again penetrate the vessels to migrate through the surrounding tissue (skeletal musculature). After this short somatic migration they incapsulate and can survive for years without undergoing further development (hypobiosis) (Dubey, 1978). Fig. 1.1. shows the life cycle of *T. canis*.

When the host becomes pregnant, the dormant larvae can then be reactivated by hormonal stimuli and migrate transplacentally to the fetus (prenatal infection) (Dubey, 1978). Larvae can also

migrate hematogenically or somatically into the mammary glands. They are then passed on to the pups with the milk (lactogenic infection).

Infective eggs which are ingested by non-canid species follow a somatic cycle similar to the one described above. This leads to the presence of larvae in tissues where they are potentially infective to predators on these animals. This type of transmission is called paratenesis (Glickman and Shantz, 1981). Possible paratenic hosts are rodents, sheep, pigs, monkeys, humans and earthworms (Bowman *et al.,* 2003). When a canid preys upon an infected paratenic host the larvae are liberated from the tissues during the digestion process and then complete their development in the intestinal tract (Glickman and Shantz, 1981).



Infection with Toxocara eggs: 1. Oral infection. 2. Tracheal migration. 3. Somatic migration.
Infection with Toxocara larvae: 4. Transplacental infection. 5. Transmammary transmission. 6. Through paratenic hosts.

Fig.1.1.: Life cycle of *Toxocara canis* (Overgaauw, 1997)

1.1.3. Pathogenesis and clinical signs in dogs

The degree of host damage and the variety of symptoms differ depending on which tissues have been invaded: the liver, lungs and central nervous system, including the eyes appear to be most sensitive. In addition the number of migrating juveniles and the age of the host are two factors that have an impact on the outcome of the infection.

Heavy prenatal *T. canis* infections cause severe discomfort in nursing pups (Bowman *et al.,* 2003). The tracheal migrating larvae cause pneumonia, associated with coughing and nasal discharge. As a result of the migration into the intestine, the puppies can show constipation or diarrhea (Singh, 2003; Rommel, 2000) with mucus and sometime hemorrhagic feces, eosinophilia, anemia, intermittent fever (Rommel, 2000), anorexia (Singh, 2003; Rommel, 2000), loss and/or hirsute hair and nervous signs (Rommel, 2000). Alarming numbers of immature and adult worms may appear in the feces or vomitus. Finally, death may result from rupture or obstruction of the intestine (Bowman *et al.,* 2003).

The infection of older or immune competent dogs appears to be clinical inapparent. Low grade infections without tracheal migration often do not cause clinical signs or only light symptoms such as diarrhea and vomitus.

1.1.4. Mode of transmission to humans

Toxocarosis is a public health problem. Humans can act as paratenic hosts in which *Toxocara* larvae will not develop but migrate and survive for a long time. The mode of transmission to humans is by oral ingestion of infective *Toxocara* eggs from contaminated soil (sapro-zoonosis), from unwashed hands, or consumption of raw vegetables (Glickman and Schantz, 1981; Glickman and Shofer, 1987). Some infections occur from ingestion of larvae in undercoocked

organ and muscle tissue of infected paratenic hosts, such as chickens, cattle and sheep (Nagakura et al., 1989; Salem and Schantz, 1992).

The dominant clinical manifestations in humans associated with toxocarosis are classified according to the organs affected. There are two main syndromes, visceral larva migrans (VLM), which encompasses disease associated with the major organs, and ocular larva migrans (OLM), in which toxocarosis pathological effects on the host are restricted to the eye and the optic nerve (Despommier, 2003).

1.1.5. Epidemiology and prevalence

T. canis is distributed worldwide (Despommier, 2003). The eggs of this parasite are very resistant to environmental adversity and remains infective for years, especially in poorly drained clay and silt soils, hence their accumulation in soil (Bowman *et al.,* 2003). It seems that soil contamination would be the most direct indicator of the risk of contacting toxocarosis by the human population (Barriga, 1988).

Children were reported to be infected more frequently than adults and VLM with more severe clinical symptoms is mainly found in children of 1 to 3 years of age (Schantz, 1989; Glickman and Schantz, 1981). This can be explained by the fact that young children often play in yards and sandpits and therefore have closer contact with potentially contaminated soil. In addition, children may often put their fingers into their mouths and sometimes eat dirt (Glickman and Schantz, 1981).

Seroprevalence for *T. canis* varies between 4.6 to 7.3% in children in the USA, 2.5% in Germany, to 83% for children in the Carribean. In tropical climates transmission is probably favored by high ambient temperature and humidity (Overgaauw, 1997).

Barriga (1998) reported a decrease in the prevalence of the infection in dogs, with increasing age of the animal and the infections are lower in well-cared pet dogs than in stray or pound dogs. The prevalence in Germany between 1999 and 2002 was given with 7.2% (Barutzki and Schaper, 2003). Furthermore, Itho *et al.* (2004) reported in Japan a prevalence of 4.3% in household dogs .

The prevalence of *T. canis* in dogs and humans is higher in tropical climates then in temperate climate regions. The prevalence in humans, for example the prevalence in Brazil in the year 2002 was 5.5% (Oliveira *et al*, 2002) and in Mexico was of 13.3%. *T. canis* was also the most prevalent species in young animals (Eguia-Aguilar *et al.,* 2005). Rubel *et al.* (2003) reported a prevalence of this parasite of 9% in a middle-income neighborhood area from Argentina and 19% in one low-income neighborhood. In Venezuela *T. canis* infections show a prevalence of 11.4% (Ramirez *et al.,* 2004). In Havana, Cuba, a prevalence of 42.2% has been reported in contaminated parks and sandpits (Dumenigo and Galvez, 1995).

Richards *et al.* (1995) reported *T. canis* in red foxes (*Vulpes vulpes*) with a prevalence of 55.9% in the United Kingdom. In Germany for the same host species, Loos-Frank and Zeyle (1982) reported a prevalence of 32%. In Greece, the prevalence is 28.6% in red foxes (Papdopoulos *et al.,* 1997).

1.2. *Ancylostoma caninum*

1.2.1. General characteristic of *A. caninum*

The hookworm *A. caninum* is a member of the family Ancylostomatidae. These parasites have a large buccal cavity directed obliquely dorsally with sharp teeth. The ventral margin of the stoma is armed with three pairs of sharp teeth (Bowman 2003; Singh, 2003).

The male measures 9-12 mm in length and is 0.4 mm wide (Singh, 2003). They have a well-developed bursa (Bowman, 2003) which contains two long and slender spicules (0.53-1-18 mm) (Singh, 2003). They can often be found in copula with the female, where the two worms form a "T" because the vulva in the female is located close to the caudal extremity, between the 2nd and 3rd part in the body (Bowman, 2003).

The female is about 15-20 mm in length and 0.6 mm wide (Singh, 2003). They lay typical strogylid eggs which appear in the feces during the morula stage of development. *Ancylostoma* eggs and larvae are destroyed by freezing. The larval forms will not develop to the infective stage at temperatures consistently below 15ºC. The optimum temperature for development is 30ºC (Bowman, 2003). Infective juveniles can survive for more than 6 months (Singh, 2003).

1.2.2. Infection route and life cycle

Infections occur through either ingestion or skin penetration by infective larvae, which then undergo more or less extensive migrations through the tissues of the host before developing into adult hookworms in the small intestine. As dogs grow older they become more resistant (Bowman, 2003).

Between 2 and 8 days are required for the morula in the hookworm eggs to develop into an infective third-stage larva (L3) (Bowman, 2003). Infective L3 attach to the canine host via skin contact, penetrate via hair follicles and eventually enter blood or lymphatic capillaries (Singh, 2003; Hawdon and Schad, 1990). They are passively carried to the pulmonary microcirculation where they can follow either of the two pathways, the "classical" or "somatic" route (Loukas and Prociv, 2001).

The “classical” route (tracheal migration) occurs when the L3 penetrates from lung tissue into alveoli, are swept up the airways with the mucus and then down into the stomach where they developed to L4. Exsheathing and further development to L5 arises in the gut (small intestine). These larvae have a primordial buccal capsule and a developed genital system that attaches to intestinal villi. They start feeding in the instestine. The large anterior glands secret various products, including proteases and anticoagulant peptides, that facilitate the blood ingestion.

The other route is the “somatic route”. After skin penetration the larvae proceed either directly to the place of infection or via the lungs into the heart. They then proceed into the systemic circulation and disperse through tissues depositing in skeletal muscle fibers as hypobiotic L3 (Loukas and Prociv, 2001) where they can survive for several years.

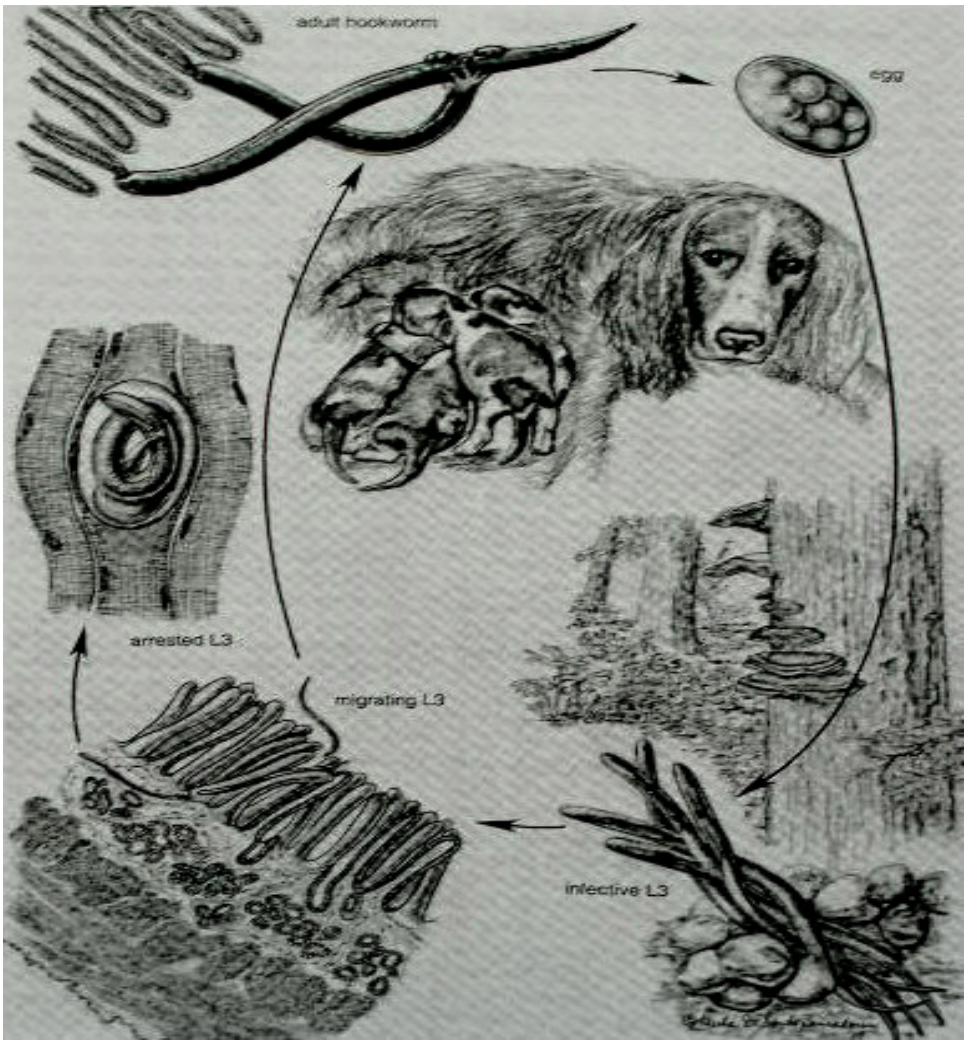


Fig.1.2.: Life cycle of *Ancylostoma caninum* (Bowman *et al.,* 2003).

The arrested larvae of *A. caninum* in the intestinal wall and skeletal muscle tissue of the adult dog population are therefore a relatively inaccessible reservoir of infection (Bowman, 2003). The larvae are continually migrating from the muscles to the intestine through the lungs. Fig. 1.2. shows the life cycle of *A. caninum*.

L3 can wander directly or through the lungs to the intestinal lumen of the adult dog and through the mammary gland to the intestines of the nursling pups (Bowman, 2003). For neonatal pups the

transmammary infection is the most important route (Loukas and Prociv, 2001). In young pups the tracheal migration predominates (Loukas and Prociv, 2001).
The prepatent period of per cutaneus infections is for puppies 1-17 days pi., and for adults 26 days pi. The prepatency period of per os infections is 15-26 days (Rommel, 2000). The eggs of *A. caninum* appear in the faeces 15-18 days after the infection in young dogs, 15- 26 days in older dogs (Singh, 2003).

A. caninum is common in dogs (Bowman, 2003; Singh, 2003, Rommel *et al.,* 2000) and many wild carnivores (Singh, 2003) such as red foxes, wolves (Eckert *et al.*, 2005; Rommel *et al.,* 2000), jackals (Eckert *et al.*, 2005; Chowdhury and Aguirre, 2001), coyote (*Canis latrans*) (Scott *et al.,* 2002; Rommel *et al.,* 2000), armadillo, spotted hyena, leopard, cheetah, african wild cat, swamp cat and fennec fox (Chowdhury and Aguirre, 2001). Insects may serve as paratenic hosts.

1.2.3. Pathogenesis and clinical signs in dogs

When the juvenile larvae penetrate the skin they produce a transitory inflammation and hyperemia (severe dermatitis). More serious changes are found in the lungs when the juveniles break into the alveoli and produce petechial hemorrhage with accumulation of blood in the terminal air cells and leukocyte infiltration resulting embarrassed respiration and lobular pneumonia (Singh, 2003).

In the intestine *A. caninum* feed on host intestinal mucosa and blood is of secondary importance (Roche and Layrisse, 1966). They enclose a plug of the mucosal lining of the intestine inside their powerful buccal capsule and this portion of the mucosa either undergoes necrotic changes or is torn away completely when the parasite changes its hold (Singh, 2003). The parasites are avid feeders of blood and it has been estimated that a single parasite may withdraw 0.36-0.80 ml blood in 24 h. Clinical signs vary in severity from asymptomatic infection to rapidly fatal exsanguination, depending on the magnitude of the challenge (determined by the virulence and

number of hookworms) and the resistance of the host. The ability to limit the number of hookworms maturing in the small intestine is influenced by age, premunition and acquired immunity of the animal affected (Bowman, 2003).

Four clinical sings for *A. caninum* infections should be distinguished:

a) peracute hookworm disease: result from the passage of infective larvae from dam to nursing pups in the milk. The pups appear healthy and sleek the first week then sickens and deteriorates rapidly in the second week. The visible mucosa is very pale and the soft to liquid feces are very dark in color due to the fact that blood, shed by the hookworms in the small intestine, has only been partially digested on the way out.

b) acute hookworm disease: results from sudden exposure of susceptible older pups to large numbers of infective larvae. Even mature dogs may be overwhelmed if exposure is sufficiently great.

c) chronic (compensated) hookworm infection: is usually asymptomatic.

d) secondary (decompensated) hookworm disease: usually involves older dogs that have more problems than just hookworms. They show anemia and are usually malnourished (Bowman, 2003).

1.2.4. Mode of transmission to humans

Humans can become infected with hookworms through ingestion of infective larvae (L3) or through direct penetration of the skin. When infective larvae penetrate the skin they undergo a prolonged migration that causes a condition known as cutaneus larva migrans (CLM). These larval migrations are characterized by the appearance of progressive, intensely pruritic, linear eruptive lesions, which are usually more extensive with *A. brazilienze* infections. When the

parasite travels it moves from a fraction of an inch to several inches a day, advancing more rapidly at night. The severity and persistence of the lesions are at least partly related to hypersensitivity resulting from a previous exposure (Bowman, 2003).

A. caninum larvae may also penetrate into deeper tissues and induce symptoms of visceral larva migrans (VLM) or migrate to the intestine and induce eosinophilic enteritis (Prociv and Croese, 1996).

1.2.5. Epidemiology and prevalence

A. caninum is distributed worldwide (Loukas and Prociv, 2001). The hookworm larvae develop well in shaded areas of well-drained soils but not in heavy, water-logged soils or where they are exposed to direct sunlight and desiccation. Moderate temperatures (23° to 30°C) and moderately moist, well-aerated medium (e.g. soil) are optimal (Bowman *et al*., 2003).

Epidemiologic studies have implicated that the presence of dogs, particularly puppies, in a household, and pica (dirt eating) are the principal risk factors for human disease. Puente *et al.* (2004) reported 34 cases of CLM in Spain and Prociv and Croese (1996) reported a series of human cases of *A. caninum* enteritic infections in Queensland, Australia, where infected humans showed eosinophilic enteritis.

Boreham and Capon (1982) reported a prevalence of *A. caninum* in parks in Brisbane, Australia of 41.7%. In Prague a prevalence of 1,4% for this parasite in children's sand pits has been reported (Valkounova, 1982), in parks in Jordan a prevalence of 5% (Abo-Shehada and Ziyadeh, 1991) and in Campo Grande, Brazil, a prevalence of 56.8% (Araujo *et al.,* 1999).

The prevalence of *Ancylostoma* spp. in dogs in Sao Paulo, Brazil, in the year 2002 was 23.6% (Oliveira-Sequeira *et al*.). In Chaco Salteno, Argentina, Taranto *et al.* (2000) reported a

prevalence of 69.8%. In Tacuarembo, Uruguay, Malgor *et al.* (1996) reported a prevalence of 96.3% of *A. caninum* in dogs during winter time. Ramirez *et al.* (2004) reported a prevalence of 24.5% for *Ancylostoma* spp in dogs under veterinary care in Venezuela. Eguia-Aguilar *et al*. (2004) reported prevalence of 62.5% for this parasite in dogs from Mexico City.

In Southern Switzerland, Deplazes *et al.* (1995), reported a prevalence of 3 and 5% in stray and abandoned dogs respectively. Barutzi and Shaper (2003) reported a prevalence of 2.8% of Ancylostomatidae in dogs from Germany. Fok *et al.* (2001) reported a prevalence of 8.1-13.1% for Ancylostomatidae in dogs from some rural and urban areas of Hungary. In Kyunggi Do, Korea, a prevalence of 26% was reported (Cho *et al.,* 1981). Ugochukwu and Ejimadu (1985) reported a prevalence of 26.77% for this parasite in Calabar, Nigeria. In South Africa the prevalence is reported with 27% (Minnaar *et al.,* 2002).

In wildlife animals, Scott *et al.* (2002) reported a prevalence of 100% for *A. caninum* in coyote (*Canis latrans*) from western Texas, USA. Currently there is no data available/published for other countries.

1.3. Diagnosis in dogs and humans

1.3.1. Diagnosis of Toxocarosis

The diagnosis of *T. canis* in dogs relies on the presence of eggs in faeces. They can be identified by flotation technique (Rommel, *et al.,* 2000).

In humans the diagnosis of *T. canis* infections is not easy, because patients do not excrete parasite material such as eggs or larvae (Overgaauw, 1997). The VLM is diagnosed primarily on the basis of clinical criteria during examination and is confirmed by immunodiagnostic test, for example the Enzyme-linked immunosorbent assay (ELISA) (Despommier, 2003). In the OLM infections

these common serological tests have a lower sensitivity due to a physiological barrier between blood and ocular fluids, the antibodies cannot be detected. A possibility to provide a definitive diagnosis of OLM infections would be the demonstration of antibodies in the vitreous humor using the ELISA TES-test or the Ouchterlony test (Overgaauw, 1997).

1.3.2. Diagnosis of Ancylostomosis

For a peracute hookworm disease in dogs the diagnosis is based in the clinical sings because the worms do not lay eggs until the 16th day of infection (Bowman, 2003). For acute infections or a chronic disease, the diagnosis relies on the presence of eggs in feces. They can be detected with the flotation technique (Rommel *et al.*, 2000). Chronic infections can be additionally detected by measurable reduction in erythrocyte count, blood hemoglobin or packed cell volume (Bowman, 2003). In humans the hookworm disease is diagnosed on the basis of clinical criteria during examination, and confirmed by immunodiagnostic tests.

1.4. Anthelmintics

Anthelmintic drugs are used to control, prevent and treat nematode and trematode parasite infections in both, humans and domestic animals (Martin *et al.,* 1997). Between 1960 and 1980, extraordinary success was achieved in the development of anthelmintic drugs for animals. In these years drugs with diverse structures, novel activity and enviable safety were produced for a global livestock industry, leading to the productivity gains needed to support a human population that grew by 1.5 billion in the same period. The next 20 years have been spent with refining existing molecules with niche activity (parasite and host specificity), improving delivery systems and worrying about the inexorable spread of drug resistance (McKellar and Jackson, 2004).
Anthelmintic research and development has demanded a very large part of the animal health pharmaceutical development effort. It is probably the only area of such research where efforts and success in animal health exceed those in human health (McKellar and Jackson, 2004).

1.4.1. Anthelmintics and their mode of action

Harder (2001) described three classes of broad-spectrum anthelmintics available. The first class is the benzimidazoles. They have been used for the control of nematode infection in ruminants, swine, horses, other food animals, in dogs, cats and in humans. The second class of anthelmintics are the imidazothiazole and tetrahydropyrimidines comprising levamisole, pyrantel, morantel and oxantel. They act as nicotinic agonists at acetylcholine-gated cation channels and are therefore quick acting at neuromuscular junctions to cause muscle contraction and paralysis of the parasites. The third class are macrocyclic lactones (milbemycins, avermectins). They show activity against nematodes and ectoparasites. They are predominantly used in livestock, however, are also recommended for the prevention of dirofilariosis in companions animals (cats and dogs). In human so far only ivermectin has been used in the control programs for onchocercosis and filariosis.
Martin *et al.* (1997) made the following anthelmintics classification for mode of action:

1.4.1.1. Agonist at nicotinic acetylcholine receptors of nematodes

The most important anthelmintics of this class are levamisole, morantel and pyrantel. The application of this anthelmintic results in the depolarization and increase in input conductance of the muscle membrane to sodium and potassium, producing depolarization and spastic paralysis of the nematode without a significant action on the host muscle. The receptor operated transmembrane ion-channel is made up of five subunits arranged around a central ion-channel like the staves of a barrel. Each subunit is made up of between 437-501 amino acid, the five subunits are two α, 1ß, 1γ and 1δ (Martin *et al.,* 1997).

1.4.1.2. Potentiate or gate the opening of glutamate gated chloride channels

This class of macrocyclic lactone (ML) contains two subclasses, the avermectins and the milbemycins. The group of avermectins includes ivermectin (IVM), abamectin, and doramectin; the group of milbemycins includes moxidectin and milbemycin oxime. The ML's are believed to open glutamate-gated chloride channels to increase the Cl- permeability of nerve and muscle membranes of invertebrates (Martin *et al.,* 1997). They were also thought to induce the release of the neurotransmitter γ-amino-butyric-acid (GABA) which interferes with neuronal transmission in parasites, but they are now known to potentiate the effect of GABA in parasitic nematodes (McKellar and Jackson, 2004).

1.4.1.3. Agonist at GABA gated chloride channels on nematode muscle (piperazine):

This anthelmintic drug opens GABA receptors on the somatic muscle of nematodes. It increases the Cl- conductance of the muscle membrane leading to an increase in the membrane potential and a reduction in excitability. This leads to a relaxation of the body muscle and flaccid paralysis. Piperazine is effective against large intestinal nematodes and is potentiated by the presence of a high pCO_2 (Martin *et al.,* 1997).

1.4.1.4. Increase the permeability of trematode tegument to calcium (praziquantel):

Their application results in a slow depolarization of the tegument and a increase of influx of calcium in *Schistosoma mansoni*. The mode of entry of calcium is unknown, but their activity may be blocked by high concentrations of magnesium, lanthanum, nickel and cobalt. The possible site of action of praziquantel appears to be in the tegument of the worm. This anthelmintic drug is used for the control of Schistosomosis and cestode infections (Martin *et al.,* 1997).

1.4.1.5. Bind selectively to parasite ß-tubulin and prevents microtubule formation:

This class of anthelmintics comprises of benzimidazoles including thiabendazole, mebendazole and fenbendazole and others. These anthelmintics bind to nematode tubulin, thus inhibiting the formation of microtubules, which are subsecuently unable to transport secretory granules or secrete enzymes within the cell cytoplasm; this eventually results in cell lysis (McKellar and Jackson, 2004). It was found that the benzimidazoles competed for the binding site on ß-tubulin with colchicine, a substance known to block cell division in the metaphase (Lacey & Gill, 1994; Martin *et al.,* 1997).

1.4.1.6. Uncouple oxidative phosphorylation

This anthelmintic class of proton ionophores includes closantel, rafoxanide, oxyclozanide and brotianide**.** These compounds are all very lipophilic so that they dissolve phospholipid membranes of cells and may shuttle across the membrane carrying protons. They do this particularly across the inner mitochondrial membrane and reduce any proton gradient. Since a proton gradient is necessary for the production of ATP by mitochondria there is an inhibition of energy production by the mitochondria following poisoning by salicylanilide (Martin *et al.,* 1997). Pax and Bennett (1989) demonstrated an effect on the tegument of *S. mansoni,* indicating that this may be a site of action for these anthelmintics. However, mitochondria may not be the only site at which these compounds exert their effect.

1.4.1.7. Inhibit glucose metabolism (diamphenethide and clorsulon)

Diamphenethide have an action which produces an elevation of malate concentration in *Fasciola hepatica* (Edwards *et al.,* 1981). Malate is an intermediary break-down product of glucose in the parasite. It is known that dopamine, a putative neurotransmitter in *Fasiola*, has a protective effect against diamphenethide (Martin *et al.,* 1997). Clorsulon is used as an anthelmintic against *F. hepatica*. It results in inhibition of the enzymes phosphoglycerate kinase and phosphoglyceromutase in the parasite (Schulman & Valentino, 1982). Thus it prevents the full breakdown of glucose by the Emden-Meyerhoff pathway and inhibits glucose utilization. The inhibition of the phosphoglycerate kinase was competitive with clorsuron inhibiting the binding of 3-phosphoglycerate and ATP to the kinase (Schulman *et al.,* 1982).

1.4.1.8. Interfere with arachidonic acid metabolism of filarial parasites and host

This anthelmintic (diethylcarbamazine) has an antagonistic action on the metabolism of arachidonic acid which is produced from the breakdown of cell membranes under the influence of phospholipase A (Maizels & Denham, 1992). In low doses this anthelmintic alters the metabolism of arachidonic acids in the host endothelial cells and microfilariae. As a result there is a constriction of the blood vessels and aggregation of the host granulocytes and host platelets. Thus it appears that diethylcarbamazine activates an innate immune response (Maizels & Denham, 1992). This mode of action could explain why diethylcarbamazine does not have an effect in vitro against the microfilariae and is not effective in non-immune animals (Martin *et al.,* 1997).

1.4.2. New anthelmintics

The intensive use of drugs for the control of nematode parasites has led and will continously lead to the development of resistance. New anthelmintics are needed for the future but the huge cost associated with the development of new compounds and the small economic size of the market for anthelmintics will make future development slow. Surveying natural substances (plants extracts and antibiotics) are also in an early stage with only a small fraction of the microbial ecosystem having been surveyed (Martin *et al.,* 1997). In the last years dioxapyrrolomycin (Hunt, 1994), paraherquamide (Schaffer *et al.,* 1990) and the anthelmintic PF1022A from the group of cyclooctadepsipeptides (Martin *et al.,* 1996) have been reported.

1.4.2.1. Dioxapyrrolomycin

Dioxapyrrolomycin appears to be a narrow-spectrum anthelmintic which works through a closantel-like mode of action The dioxapyrrolomycin, pyrrolomycin C, pyrrolomycin D, and

piericidin C2 produced by UC 11065 have been evaluated as anthelmintics against *C. elegans*, *H. contortus* and *T. colubriformis*. The dioxapyrrolomycin exhibited appreciable activity against *H. contortus* (greater than or equal to 90.9% clearance at 0.33 mg/animal in jirds), while none of the compounds showed appreciable activity against *T. colubriformis* (Conder *et al*., 1995)

1.4.2.2. Paraherquamide

The Paraherquamide compounds have been classified as diketopiperazines: marcfortine (*Penicillium roqueforti*), paraherquamide A (*Penicilium paraherquei*) and 2-desoxo-paraherquamide A (PNU-141962, semi-synthetic derivative). Its activity has been recognised since 1990 (Shoop *et al.,* 1990), its mode of action involves blockade of cholinergic neuromuscular transmission. It shows excellent activity against *H. contortus, T. colubriformis* and *O. ostertagi* (Lee *et al.,* 2002).

1.4.2.3. The Cyclooctadepsipeptide PF1022A

The Cyclodepsipeptide PF1022A, was isolated and produced by the fermentation of the fungus, *Mycelia sterilia*, belonging to the Agonomycetales and found in the microflora on the leaves of *Camelia japonica* (Sasaki *et al*.,1992). The PF1022A belongs to the class of N-methylated, 24-membered cyclooctadepsipeptides, consisting of four alternating residues of N-methyl-L-leucine, two residues of D-lactate and two residues of D-phenyllactate (Harder and Von Samson-Himmelstjerna, 2002), (Fig. 1.3).

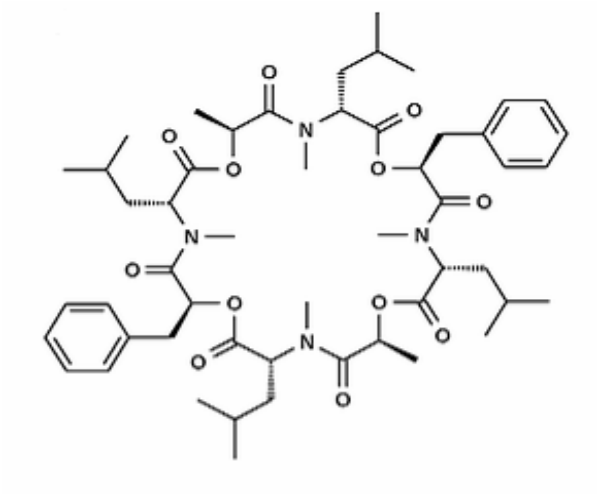


Fig. 1.3: Cyclooctadepsipeptide PF1022A structure (Harder and von Samson- Himmelstjerna, 2002).

The cyclooctadepsipeptide PF1022A has particularly attractive anthelmintic properties, its mode of activity has not been fully elucidated, although its paralytic effects have been shown not to be associated with GABAergic activity and antagonism of cholinergic receptors (Martin *et al.,* 1996). Using an *in vitro* model of *A. cantonesis*, Terada (1992) demonstrated the inhibitory effect of PF1022A on worm motility. The observed paralysis was partially antagonized by the GABAergic antagonist, picrotoxin and bicuculline, PF1022A paralysis is also antagonized by the addition of Ca^{2+}.

In studies on the potential and input conductance of the somatic muscle of *Ascaris suum*, using a two-microelectrode current-clamp technique, it was shown that PF1022A does not antagonize the effects of the selective nicotinic agonist levamisole (Martin *et al.*, 1996).

In other study, the PF1022A acts as both, an ionophore in planar lipid bilayers, similar to other cyclooctadepsipeptides, such as valinomycin and enniatin A, and as a paralysing drug in *A. suum*. However, some structurally closely related PF1022A derivatives examined in this study revealed

only an ion carrier function, such as the optical antipode PF1022-001, while other exhibit only nematocidal activity. From these results it can be concluded that the ion carrier property of the PF1022A is not responsible for its paralysing effects on nematodes (Gessner *et al*., 1996)

From the present knowledge about the mode of action of this new class of cyclooctadepsipeptides it becomes clear that they have a mode of action which is different from any of the common anthelmintics (Harder and von Samson-Himmelstjerna, 2002).

This anthelmintic has been shown to have good activity against cyathostomes (horses) (von Samson-Himmelstjerna *et al*., 2000), *T. colubriformis*, *H. contortus* (sheep) (Harder and von Samson-Himmelstjerna, 2002; von Samson-Himmelstjerna *et al*., 2000, Conder *et al.,* 1995), *D. viviparus* (cattle) (Harder and von Samson-Himmelstjerna, 2002; von Samson-Himmelstjerna *et al*., 2000), *Ostertagia ostertagi* (sheep) Conder *et al.,* 1995), *Ascaridia galli* (chicken) (Takagi *et al*., 1991), *A. caninum* (Harder and von Samson-Himmelstjerna, 2002; von Samson-Himmelstjerna *et al*., 2000) and *T. canis* (dogs), *T. cati* (cats) (Fukasche *et al.,* 1990), *Heligmosomoides polygyrus* and *Heterakis spumosa* (mice) (Harder *et al*.,1997), *Nippostrongylus brasiliensis, Strongyloides ratti* (rats) (Harder and von Samson-Himmelstjerna, 2002). The activity of PF1022A against *Angiostrongylus costarricensis* is dependent on the route of administration, the dose and formulation (Terada *et al*., 1993).

A variety of derivates of the PF1022A have been synthesised, such as modifications and exchanges of N-methyl leucine residues (Scherkenbeck *et al*.,1998), azadepsipeptides (Dyker *et al*., 2001) and thioamide analogues (Jeschke et al., 2001). The emodepside BAY 44-4400 is also a derivative of the PF1022A with the same efficacy (Nicolay *et al*., 2000). Emodepside stimulates exocytosis and elicits pharyngeal paralysis in nematodes like for example in *C. elegans* (Wilson et al., 2004).

1.5. Treatment of diseases

The anthelmintic drugs in puppies must be given between 21 and 23 days of age (Noda, 1957; Sprent 1958), because at this time most of the larvae are in the digestive tract before reaching adulthood and fertility (Sprent, 1958). Repetition of the treatment every 14 days will eliminate the new worms and prevent new infection for bitch´s milk for at last 38 days after whelping (Zimmerman *et al.*, 1985).

In theory, 4 treatments of the new pups at 2, 4, 6 and 8 weeks of age should prevent the establishment of transuterine or milk infections. Reactivation of the dormant larvae in the bitch by late pregnancy or lactation often results in a temporary patent infection of the bitch herself. The intestinal parasites in the bitch start laying eggs after 24 days of whelping and continue doing so for between 2 weeks up to a few months (Douglas and Baker, 1959). The anthelmintic treatment of the mother at the same time as the pups is recommended (Barriga, 1988).

The following anthelmintics are usually used against *T. canis* for puppies over 2 and 3 weeks of age: pyrantel (26,8 mg/kg body weight) (Barr *et al.,* 1998;) and febantel (26,8 to 35.2 mg/kg body weight) (Barr *et al.,* 1998) and with praziquantel (5,4-7.0 mg/kg body weight) (Barr *et al.,* 1998) . Several preparations of ivermectin (6µg/kg body weight) (Venco *et al*., 2004) are also available for puppies and are normally used, when mixed infections with *Ancylostoma* occur, but these products are not approved for dogs younger than 4 weeks (Bowman *et al.,* 2003).

The administration of anthelmintic drugs in puppies against hookworm (*A. caninum*) should begin 2 weeks after pups are whelped and continue for 3 months. The bitches should be treated with fenbendazole (50 mg/kg body weight) from the 14th day of gestation to the 14th day of lactation to prevent further losses (Bowman, 2003). This treatment attacks the reactivated larvae and is effective but also quite expensive. It also has been shown that ivermectin treatment of the bitch (0.5 mg/kg body weight) administered 4 to 9 days before whelping followed by a second

treatment 10 days later can also prevent puppies from being infected by larvae passed in the milk (Bowman, 2003).

Against *A. caninum* are furthermore used: pyrantel pamoate, diclorvos, mebendazole, febantel, fenbendazole, milbemycin oxime and nitroscanate. For the peracute neonatal hookworm disease a blood transfusion is essential to keep affected pups alive long enough for anthelmintic medication to take effect. In acute and chronic hookworm infection, response to simple anthelmintic therapy is usually dramatic. Supportive therapy beyond provision of an adequate diet is unnecessary. In case of secondary hookworm disease is necessary to give a supportive therapy and the administration of at least one anthelmintic (Bowman, 2003).

Prevention of environmental contamination with dog's feces is one important point for the parasitosis decrement in dogs and to prevent infection in humans. An average size dog passes 136g of faeces per day (Feldman, 1974). A light *T. canis* infection can result in 10,000 parasite eggs per gram feces (Vaughn and Murphy, 1962). For this reason the recollection of dog's feces is very important to prevent the infection from other dogs and humans, especially from children in playgrounds, sandboxes or gardens. Regular cage/cannel cleaning, run sanitation and periodic anthelmintic medication of all adult dogs are essential to reduce the level of environmental contamination with larvae.

1.6. Anthelmintic resistance (AR)

Widespread treatment of farm animals with anthelmintics has been practiced for many years to prevent disease and death and increase productivity. The first reports of AR were to the drug phenothiazine in the late 1950s and early 1960s, first in *H. contortus* of sheep (Kaplan, 2004). In 1961, thiabendazole was introduced as the first anthelmintic that combined efficacious broad-spectrum nematocide activity with low toxicity. The rapid acceptance and widespread use of thiabendazole and then other benzimidazole anthelmintics marked the beginning of the modern

chemical assault on helminth parasites. However, within a few years, resistence to thiabendazole was reported, again first in the sheep nematode *H. contortus* (Conway,1964). In the mid-1970s the resistence to benzimidazole anthelmintics was common and widespread in both sheep and horses throughout the world. The appearance of anthelmintic resistance to newer anthelmintic drug classes such as imidazothiazole, tetrahydropyrimidines and macrocyclic lactones, introduced after 1970 followed the same pattern. In the early 1980s, reports of multiple-drug resistant (MDR) worms appeared for the first time.

In the 1990s widespread reports of MDR worms, including resistance to macrocyclic lactone anthelmintics, had elevated the issue of anthelmintic resistance from being one of academic interest to being a major threat to livestock production in many areas of the world (Kaplan, 2004).

In the last years an important and increasing problem of drug resistance is observed to all the major groups of anthelmintics (Albonico *et al.,* 2004).

AR is defined as a heritable reduction in the sensitivity of a parasite population to the action of a drug. This is expressed as the decrease of the frequency of individual parasites affected by exposure to the drug, compared to the frequency observed in the same population upon initial or prior to exposure (Conder and Campbell, 1995).

In the last years AR has also been suspected in humans: to mebendazole in *Necator americanus* in Mali (De Clercq *et al.,* 1997) and to pyrantel (P) in *Ancylostoma duodenale* in Northwestern Australia (Reynoldson *et al.,* 1997). Resistance against oxamniquine in schistosomes in Brazil has been reported by Cioli (Cioli *et al.,* 1993; Cioli *et al.,* 1992) and resistance to praziquantel is believed to be present in Egypt (Ismail *et al.,* 1999). Resistance has also been reported to IVM in onchocercosis (Shoop 1993; Geerts *et al.,* 1997) and to praziquantel in *Schistosoma mansoni* in Senegal (Fallon and Doenhoff, 1994; Geerts and Gryseels, 2000). Some studies and models indicate that the low cure rates to praziquantel in *S. mansoni* in Senegal may be explained by the

specific epidemiological situation in the northern region of the country (high initial worm burdens and high transmission intensities) (Geerts and Gryseels, 2001).

Resistance against the common anthelmintics in animals is a problem, principally in sheep, goat and horse parasites (Coles 2003; Sangster, 2001). In small rumiants, serious problems with AR were reported in some humid tropical areas in *Haemonchus contortus* (Dorny *et al.,* 1994). Beveridge *et al.* (1990) reported high levels of BZ resistance in *H. contortus, Nematodirus* spp*., Teladorsagia* spp. and *Trychostrongylus* spp. in sheep in South Australia.

In sheep, Van Wyk *et al.* (1999) and Waller *et al.* (1996) reported AR to BZ, IVM and levamisole in nematodes of small ruminants in South Africa, Brazil, Paraguay and Uruguay. Sangster (1996) reported resistance to ivermectin in *H. contortus* in South Africa, Brazil an in the USA and in *T. colubriformis* in Australia and levamisole for *T. colubriformis* and *Teladorsagia (Ostertagia) circumcincta* in sheep and goat.

Conder and Campbell (1995) reported resistance to IVM in nematodes of small ruminants. Sangster and Grill (1999) reported resistance in the same animal species to benzimidazole, imidazole, naphtalophos and macrocyclic lactones (ML) in trichostrongylids. In addition, AR to IVM in goats has been reported for *T. columbriformis* (Prichard, 1999).

Resistance to BZ, IVM and P in *Oesophagostomum* spp. in pigs has also been reported. In horses resistance to BZ, phenothiazine (PH), piperazine and P to small strongyles and in cattle resistance to BZ and ML to trichostrongylids (Sangster and Grill, 1999) have been found. Evidence of resistance of *Fasciola hepatica* to triclabendazole has been reported in sheep from Australia, Ireland and Scotland (Coles, 1999; Jackson and Coop, 2000). Recent reports in Brazil, Argentina and New Zealand suggest an increase in the AR of *Cooperia* spp. to ML's in cattle (Kaplan, 2004).

Drug resistance can arise in a number of ways:

- a change in the molecular target, so that the drug no longer recognises the target and is thus ineffective,
- a change in metabolism that inactivates or removes the drug, or that prevents its activation,
- a change in the distribution of the drug in the target organism that prevents the drug from accessing its site of action (Wolstenholme *et al.,* 2004). For Köhler (2001) this mechanism is the most important one in the development of AR.
- amplification of target genes to overcome drug action (Wolstenholme *et al.,* 2004).

It was proposed that the chemistry of the different classes of antiparasite drugs could be altered to get around the mutation in the site of action receptors (Genetic resistance) (Prichard, 1994). The number of genes involved in AR and their dominance or recessiveness are other factors with an important impact on the rate at which AR spreads (Anderson *et al.,* 1998).The high genetic diversity of parasitic helminths, coupled with their large populations, increases the likelihood that resistance alleles will already be present in a population, possibly at relatively high frecuency (Wolstenholme *et al.,* 2004). For example, the resistance to BZ in *H. contortus* is polygenic with at least two, possible three genes with recessive alleles involved. Levamisole resistance seems to be caused by one gene or gene cluster with autosomal recessive alleles. IVM resistance appears to be mediated by a single gene or gene complex with primarily dominant effects (Anderson *et al.,* 1998).

The AR to benzimidazoles (BZ) can be explained by loss of susceptible phenotypes of the ß-tubulin and the survival of resistance phenotypes (Martin *et al.,* 1997). Ross *et al.* (1995) has shown that there is a reduction in the number of isotype alleles for ß-tubulin during the appearance of BZ resistance in *H. contortus*.

In *H. contortus,* the well-known Phe-Tyr polymorphism at codon of ß-tubulin isotype 1 was the first described and has frecuently been considered the most important mutation conferring resistance to BZ (Kwa *et al*., 1994). The 200 codon polymorphism has been described in cattle nematode, *C. oncophora* and found to occur in BZ-resistant populations (Silvestre and Cabaret, 2002; Njue and Prichard, 2003). In horse cyathostomins, the ß-tubulin isotype 1 codon 200 polymorphism ist not the only, and probably not even the most important, mutation with respect to resistance (Pape *et al.*, 2003).

In hightly resistant populations of *H. contortus* were also known to posses a delection in ß-tubulin isotype 2. A second Phe-Tyr polymorphism, at codon 167 of ß-tubulin isotype 1, was detected in BZ-resistant populations of *H. contortus.* The same two polimorphisms also occur in the ß-tubulin isotype 2 gene of *H. contortus,* and they too can confer BZ resistance (Prichard, 2001). The same polymorphism was present in BZ-resistant *Teladorsagia circumcincta*, but not in *Trichostrogylus colubriformis* (Silvestre and Cabaret, 2002).

The resistance to triclabendazole does not appear to be associated with mutations in ß-tubulin (Robinson *et al.,* 2002). But there is a possibility that it enhances the metabolism of triclabendazole, in that resistant flukes can metabolize triclabendazole sulfoxide (TCBZ.SO) to the relatively inert sulfone metabolite to a great extent than can their susceptible counterparts (Robinson *et al.,* 2004).

Resistance occurs more frequently when the same anthelmintic agent has been used intensively and without good hygiene standards to assist control (Martin *et al.,* 1997). Within a population of nematodes exposed regularly to a drug this will confer an evolutionary advantage to the worms with the altered genome (Pape *et al.,* 2003). A lot of anthelmintic drugs have been used continuously for parasite control and resistance has gradually developed. It is now found in a number of parasite species against most of the anthelmintics (Prichard, 1994).

Geerts and Gryseels (2001) propose the following guidelines to delay the development of AR:

- Give priority to accessible diagnosis and treatment of symptomatic individual cases
- use community-based treatment if only really necessary
- avoid indiscriminate mass treatment
- reduce treatment frequency by combining the use of drugs with other measures such as health education programs between others
- avoid exposure of the whole helminth population to the drug
- use correct dose, implement combined drug use or annual rotation of drug and monitor the development of drug resistance.

1.7. Molecular research methods

1.7.1. General aspects

The phylum Nematoda is ecologically and biologically extreme diverse. Phylogenetic analysis is a useful tool to answer question pertaining to the evolution of nematodes, for example if pre-adaptation is necessary in the evolutionary trait.

The molecular phylogenetic analysis methods allow a relatively unbiased view of the phylogenetic structure, the testing of several hypothesis and in addition it promises insights into many aspects of the phylum Nematoda. Once the evolutionary framework is in place, the search for genes and processes unique to and/or important in parasitism can be followed up. These techniques also allow the analysis of populations or co-generic species, the relationship of nematode phyla or orders (Blaxter, 2001).

Before the advent of molecular techniques the systematic studies of this phylum was hindered by a number of factors:

- the number of species involved
- the morphological diversity
- the limitations of older techniques such as light microscopy
- the inevitable specialization of nematodes systematics.

Molecular phylogenetic markers offer a solution to some of these problems. Establishing the genetic structure of a nematode species population has important implications for understanding the epidemiology, evolutionary processes such as adaptation to host defence systems and the development of drug resistance (Blouin, 1998; Viney, 1998).

In the history of nematode molecular research, a number of different genes have already been used for analysis: cytochrome *c* (Vanfleteren *et al.,* 1994), globin (Blaxter *et al.,* 1994; Vanfleteren *et al.,* 1994), RNA polymerase II (Baldwin *et al.,* 1997), heat shock protein 70

(Snutch and Baillie, 1984; Beckenbach *et al.,* 1992), ribosomal RNAs and their spacer segments (Aleshin *et al.,* 1998; Blaxter *et al.,* 1998; Kampfer *et al.,* 1998; Dorris *et al.,* 1999) and mitochondrial genes (Hyman and Slater, 1990; Anderson *et al.,* 1993; Pelonquin *et al.,* 1993; Powers *et al.,* 1993; Grant, 1994; Hyman and Beck Azevedo, 1996; Blouin *et al.,* 1997; Hugall *et al.,* 1997; Keddie *et al.,* 1998).

The slow evolving genes (cytocrome *c,* globin, coding regions of ribosomal RNAs, RNA polymerase II and heat shock protein 70) are suitable for the analysis of deep events in nematode evolution, while the mitochondrial and ribosomal spacer genes are more suitable for intra-species, intra-genus and intra-family analyses (Blaxter, 2001).

In *T. canis* and *A. caninum* molecular studies are focused on the secreted proteins of the migrating juvenile stages, present in the cuticle (Doedens *et al.,* 2001), for example mucins (Page *et al.,* 1992; Maizels *et al*., 2000; Gems and Maizels, 1996), cystein (Falcone *et al*., 2000), lectins (Maizels *et al*., 2000), between others, and phylogenetic identification with taxonomic propose (Blaxter, 2001; Chilton and Gasser, 1996; Gasser *et al.,* 1996; Mitreva *et al*., 2005; Tetteh et al., 1999), but for these parasites have been no reported studies about the presence of a homologue receptor of a latrophilin-like receptor (HC110-R) identified in *H. contortus*

1.7.2. Internal transcribed spacers (ITS)

All regions of the nuclear and mitochondrial genomes of parasites accumulate mutations over time, and some regions are more accessible to nucleotide changes than others. If the target region should provide genetic markers for the identification of species, then the level of within-species variation in the sequence should be substantially lower than the degree of variation between or among species (Gasser, 2001).

Nuclear rDNA is a useful target for the definition of species or strain markers. These sequences exhibit patterns of “concerted evolution”, which result in sequence similarity tending to be

greater within a species than between species. Consequently it can provide useful genetic markers for parasites species (Gasser, 2001). Some studies have demonstrated that internal transcribed spacers (ITS-1 and ITS-2) of rDNA provide accurate species markers for a range of bursate nematodes which can be utilized to develop PCR-based diagnostic system (Epe *et al.,* 1997; von Samson-Himmelstjerna *et al.,* 1997; Höglund *et al.,* 1999).

ITS-2 has been used to determinate seven taxa of the Ancylostomatoidea (Blaxter, 2001). Chilton and Gasser (1998) described the sequences two rDNA ITS (1 and 2) genes of the hookworms *A. tubaeforme, A. ceylanicum* and *A. duadenale.* The sequence lengths were similar among the four species, except that in *A. ceylanicum* it was slighty longer (by 5-7 bp). The predicted secondary structure of the ITS 2 precursor rRNA was similar for all species. PCR-based restriction fragment length polymorphism (PCR-RFLP) and PCR-linked single strand conformation polymorphism of the rDNA region comprising the ITS-1 and ITS-2 and intervening 5.8S rRNA gene have been used to distinguish among some species of hookworm from carnivores (Gasser *et al.,* 1996; Monti *et al.,* 1998; Romstad *et al.,* 1998; Gasser *et al.,* 1998).

Jacobs *et al.* (1997) extracted genomic DNA from ascaroid nematodes from dogs, foxes and cats and a region spanning the ITS-2 of the rDNA were amplified by PCR and PCR-linked RFLP assay. Representative ITS-2 for *T. caninum, T. cati* and *Toxascaris leonina* were sequenced. The three species could be differentiated from each other and from other ascaridoids that may be found in human tissues by use of two endonucleases, *Hinf I* and *Rsa I.*

Ribosomal DNA (rDNA) is therefore useful for analyzing genetic variation within and among parasite populations (Gasser, 2001), although some species can display low levels of intraspecific variation (Anderson *et al.,* 1998).

Other regions of the genome, such as repetitive elements, also evolve rapidly and can be exploited as polymorphic markers in random or selective PCR assays. Characterisation of such

markers is of significance for the construction of genetic linkage maps for nematode species (Grant, 1994; Roos *et al.,* 1998).

1.7.3. Molecular studies of the HC110-R receptor

The HC110-R receptor is a latrophilin-like receptor identified in *H. contortus* and belongs to a class of g-protein coupled receptors (GPCR's) (Fig. 1.4). The HC110-R shows structural similarities to the mammalian g-protein-coupled receptor latrophilin and there are reports that α latrotoxin (αLTX), a component of black widow spider venom, activates the HC110-R receptor in *H. contortus* (Saeger *et al*., 2001). This transmembrane receptor is also present in other species of nematodes.

The heptahelical transmembrane protein of 1,014 amino acids (B0457.1) of the nematode *C. elegans* seems to be the orthologue receptor to HC110-R (Saeger *et al*., 2001). The B0457.1 receptor of *C. elegans* was identified also as a orthologue of latrophilin, a g-protein-coupled receptor. The α LTX is a potent toxin and lethal for *C. elegans.* Therefore latrophilin might have a primary role in mediating the effects of LTX in mammals (Mee *et al.*, 2004). The g-protein coupled receptors, make 5% of the genome in *C. elegans* (Bargmann,1998). The g-proteins are involved in the transduction signals in the majority of eukaryotic cell types (Knippers, 2001).

Other putative G-protein coupled receptors were identified recently in *Cooperia oncophora* and *Ostertagia ostertagi* as putative orthologues of the HC110-R of *H. contortus* (Welz *et al*., 2005).

The activation of a G protein-coupled receptor latrophilin and a single-transmembrane receptor neurexin by αLTX, the major component of black widow spider venom follows two parallel mechanisms:

A. Ca^{2+} dependent effect: the effect is due to this cation entering terminals through the toxin pore (Khvotchev and Sudhof, 2000; Ashton *et al.,* 2001). The pores formed by latrotoxins in the plasma membrane of receptor expressing cells are permeable to Ca^{2+}, influx of extracellular Ca^{2+} into the cytosol of excitable cells can stimulate exocytosis directly (Krasnoperov *et al.,* 1997; Sugita *et al.,* 1999). Toxin pores can also allow passage of small compounds, that are normally present in the cytoplasm as norepinephrine, acetylcholine, GABA or glutamate, these pores cause massive leakage of the transmitter pool present in the cylosol (Davleton *et al.,* 1998; Ashton *et al.,* 2001). The pore formation can be mediated from receptor mediated signalling (Khvotchev and Sudhof, 2000; Ashton *et al.,* 2001).

B. Ca^{2+} independent effect: (Khvotchev and Sudhof, 2000; Ashton *et al.,* 2001), is especially distinct in motor nerve terminals (Fesce *et al.,* 1986), although central synapses can also be stimulated by this toxin in the nominal absence of Ca^{2+} (Capogna *et al.,* 1996) but presence of Mg^{2+} (Magazanik *et al.,* 1992).

The LTX the ligand of latrophilin, binds to the amino terminus of HC110-R and induces influx of external Ca^{2+} through Cd^{2+} - and nifedipine-blockable Ca^{2+} channels in HC110-R transfected HEK-293 cells. The PF1022A binds to the extracellular amino-terminal region of HC110-R and acts as an antagonist to LTX signaling in HC110-R transfected HEK-293 cells (Saeger *et al.,* 2001).The natural ligand to the HC110-R is unknown, the identification of this will reveal which physiological signaling pathways are impaired in nematodes by PF1022A.

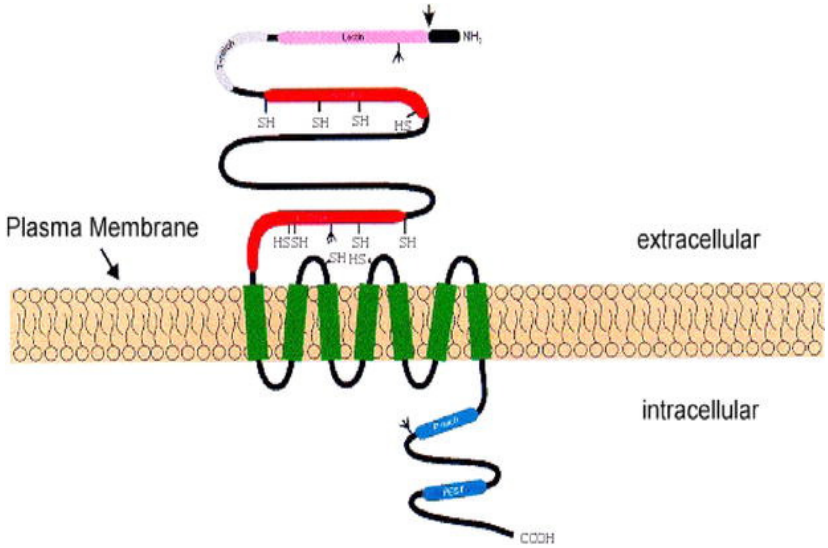


Fig. 1.4.: Schematic view of HC110-R disposition in plasma membrane (Saeger *et al*., 2001)

The new anthelmintic drug emodepside belongs also to the class of cyclooctadepsipeptides and was identified as a ligand of the nematode latrophilin (Saeger *et al.,* 2001). In the pharyngeal nervous system of *C. elegans*, emodepside stimulates exocytosis and elicits pharyngeal paralysis (Wilson *et al*., 2004).

Emodepside causes paralysis in nematodes and bind to the amino-terminus of Hc110-R and affect latrotoxin signaling through Hc110-R. Thus, emodepside impaired the latrotoxin-induced Ca^{2+} response in a concentration-dependent way. Emodepside acts as an antagonist to latrotoxin signaling by impairing the influx of external Ca^{2+} (Harder *et al*., 2003) and fuctional studies indicate that the predominant target site of emodepside is presynaptic (Wilson *et al.,* 2003).

2. Materials and methods

2.1. Material

2.1.1. Chemicals and equipment

2.1.1.1. Kits

- QuickPrep™micro mRNA Purification Kit: Amersham Biosciences, Freiburg
- Topo TA Cloning® Kit: Invitrogen™ Life Technologie, Karlsruhe, Germany
- BD SMART™RACE cDNA Amplification Kit: BD Biosciences Clontech, U. S. A
- 3` RACE System for Rapid Amplification of cDNA ends: Invitrogen™ Life Technologie, Karlsruhe, Germany
- Lambda ZAP® II Predigested *Eco*RI/CIAP-Treated Vector Kit: Stratagene, Heidelberg
- PCR DIG Probe Synthesis Kit: Roche Applied Science, Penzberg
- Gigapack® III Gold Packaging Extract: Stratagene, Heidelberg
- DIG Wash and Block Buffer Set: Roche Applied Science, Penzberg
- NucleoSpin®Plasmid Purification Kit: Macherey & Nagel, Düren
- NucleoSpin® Tissue DNA Isolation Kit: Macherey & Nagel, Düren
- NucleoBond®Plasmid Purification Kit: Macherey & Nagel, Düren

2.1.1.2. Autoradiographic materials

- KODAK GBX Fixer and Replenisher: KODAK, Rocherster, N. Y., U. S. A
- KODAK GBX Developer and Replenisher: KODAK, Rocherster, N. Y., U. S. A
- Lumi-Film Chemiluminescent Detection Film: Roche Applied Science, Penzberg
- Autoradiography Cassette RPN 1645 35 x 43 cm: KODAK, Rocherster, N. Y., U. S. A

2.1.1.3. Screening and chemiluminescent detection

- CSPD-Star, ready-to-use: Roche Applied Science, Penzberg
- Anti-DIG-Antibody Conjugates (Anti-Digoxigenin-Ap, Fab fragments): Roche Applied Science, Penzberg
- DIG Easy Hyb: Roche Applied Science, Penzberg
- Nylon membranes for colony and plaque hybridization: Roche Applied Science, Penzberg
- Hybridization bags: Roche Applied Science, Penzberg

2.1.1.4. Marker

- DNA Molecular Weight Marker II, DIG-labeled: Roche Applied Science, Penzberg
- Low Mass DNA Ladder: Invitrogen™ Life Technologie, Karlsruhe, Germany
- 250bp Ladder: Invitrogen™ Life Technologie, Karlsruhe, Germany
- 100 bp Ladder plus, Gene Ruler™: MBI Fermentas,Vilnius, Litauen
- Lambda DNA/*Eco*RI + Hind lll Marker: Fermentas,Vilnius, Litauen

2.1.1.5. Medium and agar

- Agarose NEEO: Roth, Karlsruhe
- Agar N.1 Oxoid®, England
- NZY Broth Medium: Lab M™

2.1.1.6. Other reagents

- Trizol® Reagent: Invitrogen™ Life Technologie, Karlsruhe, Germany
- Enzyme *Eco*RI: Promega, Madison, U. S. A
- Chloroform and Phenol-Chloroform: Roth, Karlsruhe
- IPTG: Roth, Karlsruhe
- Xgal: Roth, Karlsruhe
- Proteinase K: Sigma, Saint Louis, Missouri, U. S. A
- Formamide: Sigma, Saint Louis, Missouri, U. S. A
- Gelstar®: Nucleic Acid Gel Stain, Cambrex
- Ampuwa®: Fresenius, Bad Hamburg

2.1.1.7. Supplies

- Amicon Microcon® YM-100: Amicon, Beverly, U.S.A
- Petri dishes, Ø 9cm and 150mm: Renner, Dannstadt
- Adjustable pipettors 10 µl,100 µl , 1000 µl und 5000 ml : EPPENDORF, Hamburg
- Microcentrifuge tubes 0,2 µl ; 1,5 ml; 2 ml: Biozym, Hessisch- Oldendorf
- Sterile, disposable 15 ml, 50 ml graduated conical tubes: Renner, Dannstadt

2.1.1.8. Software

- Program Align™ Plus: Sequence Alignment Program Version 4.0, S & E SOFTWARE
- Chromas, version 1.45: Connor McCarthy, Griffith University
- Microsoft Word, Excel, Office, Microsoft Inc. U. S. A

2.1.1.9. Solutions and buffer

High salt buffer:	10 mM TrisHCl, pH 7.5; 1mM EDTA; 0.5 M NaCl
Low salt buffer:	10 mM TrisHCl, pH 7.5; 1mM EDTA; 0.1 M NaCl
Elution buffer:	10 mM TrisHCl, pH 7.5; 1mM EDTA
Glycogen solution:	5-10 mg/ml in DEPC-treated water
K acetate solution:	2.5 M potassium acetate
Denaturation solution:	1.5M NaCl; 0.5M NaOH in H_2O
Neutralization solution:	1M TrisHCl, pH 7.4; 1.5M NaCl in H_2O
LB-Medium:	1%NaCl; 1%Tryptone; 0.5% yeast extract in H_2O, pH 7.0
LB-agar:	LB medium with 1.5% Agar
NZY-medium:	5 g NaCl; 2 g $MgSO_4$ x 7 H_2O; 5 g yeast extract;10g NZ amine, ad 1L H_2O, pH 7.5
NZY-top agar:	NZY-medium with 0.7% agarose
SM buffer:	100 mM NaCl; 8 mM $MgSO_4$ x 7 H_2O; 50 mM TrisHCl, pH 7.5; 0.01% gelatin in H_2O
SSC 20X buffer:	3 M NaCl; 0.3 M sodium citrate, pH 7.0 in H_2O
TAE-buffer 40X:	1.6 M Tris; 1.33 M sodium acetate; 0.04 M EDTA in H_2O, pH 8,0
TE-buffer:	10 mM TrisHCl, pH 7.5-8.0; 1 M EDTA in H_2O
10X MOPS-buffer:	200 mM morpholineopropanesulfonic acid (MOPS); 50mM sodium acetate; 20mM, EDTA to pH 7.0
Loading dye:	0.25 % Bromophenolblue; 40 % Saccharose, in double distilled water
Low stringency buffer:	2 x SSC; 0.1% SDS
High stringency buffer:	0.1 x SSC; 0.1% SDS

Maleic acid buffer:	0.1 M Maleic acid; 0.15 M NaCl; adjust with NaOH to pH 7.5
Washing buffer:	0.1M Maleic acid; 0.15 M NaCl, pH 7.5; 0.3% (^{V}/v) tween 20
Detection buffer:	0.1 M Tris HCl; 0.1 M NaCl, pH 9.5
DEPC-bidest:	0.1 % Diethylpyrocarbonate in double distilled water, 12 hours shaking at 37 °C, finally 15 min at 100°C

2.1.2. Bacterial strains and vectors

2.1.2.1. Bacterial strains:

-*E.coli* XL 1-Blue	Δ (mcrA), 183, Δ (mcrCB-hsd SMR-mrr) 173, endAl, supE44, thi-1, recA1, gyrA96, re1A1, lac, [F` proAB, lacI^{q} ZΔM15] (Bullock *et al*., 1987).
-*E.coli* SOLR™	e14` (mcrA), Δ (mcrCB-hsdSMR-mrr) 171, sbcC, recB, recJ, umuC: TN5 (Kan$^{r}$), uvrC, supE44, lac, gyrA96, relAl, thi-1, endAl, λ$^{r}$, [F` proAB, lacI^{q} ZΔM15] (Hay and Short, 1992).
-*E.coli* TOP10	F ¯mcrA Δ (mrr, hsdRMS-mcrBC) Φ80lac, ZΔM1 ΔlacX74 recA1 deoR araD139 Δ (ara-leu) 7697 galU galK rpsL (Str^{R}) endA1 nupG

2.1.2.2. Vectors:

-pBluescript® II SK (+/-) (Short *et al*.,1988)

-pCR® 4-TOPO®

2.1.2.3. Helper phage:

-ExAssist™ (Hay and Short, 1992)

2.1.3. Parasites

2.1.3.1. *Toxocara canis*

Adult parasites of the genus *Toxocara canis* were obtained from

a) dogs, experimentally infected and euthanized in the diagnostic section of the "Institut für Parasitologie, Tierärztliche Hochschule Hannover".
b) deep frozen (-80°C) samples given by Bayer® AG Germany

The parasites were aliquoted in sterile H_2O and stored at -80 °C.

2.1.3.2. *Ancylostoma* spp.

Adult parasites of the genus *Ancylostoma caninum* were obtained from dogs experimentally infected and euthanized in the diagnostic section of the "Institut für Parasitologie, Tierärztliche

Hochschule Hannover". The used *Ancylostoma tubaeforme* were deep frozen samples from the institute.
The parasites were aliquoted in sterile H_2O and stored at -80 °C.

2.2. Methods

2.2.1. Nucleic acid-analysis

2.2.1.1. Quantitation of nucleic acid

The concentration of nucleic acid was determined by spectrophotometry (Gene Quantpro RNA/DNA Calculator, Amersham Pharmacia Biotech, Freiburg). RNA and DNA can absorb UV light by 260 nm, the concentration can be determined using the following formula:

$$DNA = A_{260} \times 50\ \mu g/ml$$

$$RNA = A_{260} \times 40\ \mu g/ml$$

The OD value should be between 0.1 and 1.0 to ensure an optimal measurement and the purity can be determined using the ration: A_{260}/A_{280}, for DNA, the optimal purity ratio is between 1.9 - 2.1.

2.2.1.2. Isolation of total-RNA

For the total-RNA isolation, approximately 100 mg of adult *T. canis* worms was homogenised using a pestle in 1 ml of Trizol reagent (Invitrogen Life technologies, Karlsruhe, Germany), a

mixture of Phenol and Guanidinium thiocyanate (Chomczynski and Sacchi, 1987).The homogenate was incubated 5 minutes at room temperature and vortexed for 15 seconds.

The mixture was stored 5-15 minutes at room temperature and centrifuged at 13000 rpm (Biofuge^{pico}, Heraeus Holding GmbH) for 15 minutes at 4°C. After centrifugation the upper aqueous phase was separated (RNA) and transferred to a fresh tube. The RNA was precipitated with 100% isopropanol and incubated at room temperature for 10 minutes. It was then centrifuged at 13000 rpm for 30 minutes and the pellet washed with 75% ethanol and resuspended finally in RNase-free water by diethylpyrocarbonate (DEPC). The total-RNA was stored at - 80°C.

2.2.1.3. Isolation of polyadenylated RNA (poly (A^+) RNA)

The isolation of poly (A^+) RNA, was performed using the QuickPrep^{TM} *micro* mRNA purification Kit (Amersham Biosciences)**.** The worms were homogenised, with a manual tissue homogeniser, in 400 µl of extraction buffer and diluted with 2 volumes of elution buffer. It was then centifuged at 13000 rpm (Biofuge^{pico}, Heraeus Holding GmbH) for 1 minute.

For binding, the cleared homogenate was transferred to one tube with the pellet of oligo (dT)-cellulose (resin), resuspended by manual inversion for 3 minutes and centrifuged for 10 seconds at 13000 rpm. To remove other contaminants (proteins, nucleic acids and carbohydrates) the oligo (dT)-cellulose was washed to the following treatment: it was resuspended manually with high salt buffer and centrifuged for 10 seconds at 13000 rpm for 5 times and 2 times with lower salt buffer. Then the resin was resuspended in 0.3 ml low salt buffer and transferred to a microspin columm, centrifuged at 13000 rpm for 5 seconds. Then the effluent was removed and the columm was additionally washed 2 times with 0.5 ml of low salt buffer. The mRNA was removed from the columm by eluting 2 times with 0.2 ml prewarmed elution buffer by centrifugation.

The mRNA was concentrated by precipitation. For precipitation 10 µl of glycogen solution were added, as well as 40 µl of K acetate solution and 1 ml 95% ethanol (chilled to -20°C). The sample was stored at -80°C overnight. The next day it was centrifuged at 13000 rpm for 30 minutes and the pellet was washed with 75% ethanol and resuspended finally in DEPC-(diethylpyrocarbonate) treated water and then the mRNA was stored at -80°C.

2.2.2. RNA-electrophoresis

2.2.2.1. RNA in formaldehyde gel

Gel electrophoresis was done to verify the quality and stability of RNA with formaldehyde according to modifications of Lehrach *et.al* (1977) and Goldberg (1980), and with formamide (Staynow, 1972, Spohr *et al.* 1976).

The 1% agarose gel was prepared with 0.1% DEPC-H_2O and cooled down to max. 60°C. The following components were added: 10X MOPS running buffer, 37% formaldehyde (12.3 M) and then the gel-solution was finally poured into the plastic sledge. To run a RNA sample on the gel nuclease-free water was added to bring the sample volumes to 11µl and mixed with the following components:

RNA	1 µg in 11 µl
10X MOPS running buffer	5.0 µl
12.3 M formaldehyde	9.0 µl
Formamide	25.0 µl

The samples were heated at 55°C for 15 minutes and 10 µl of loading dye (1mM EDTA, 0.25% bromophenol blue, 50% glycerol, 60 µg/ml ethidium bromide) was added. The samples were run in a electrophorese-set-up in a Horizontal Mini Submarine Unit (Hoefer He 33,), at 5V/cm, until

the bromophenol blue had been migrated one-half to two-thirds of the length of the gel. The gel was visualised on a UV light transilluminator (UV-Table TF-M 20x40cm, 312nm; INTAS, Göttingen).

2.2.3. DNA Preparation

2.2.3.1. cDNA synthesis

The cDNA, used in the Polymerase Chain Reactions (PCR), was prepared with the cDNA synthesis Kit of the 3`RACE System for Rapid Amplification of cDNA ends Kit (Invitrogen™ Life Technologie, Karlsruhe, Germany).

Approximately 50 ng of poly($A^{\pm}$) RNA was combined with DEPC-treated water to reach a final volume of 11 µl in a 0.5 ml microcentrifuge tube. 1 µl of 10 µM adapter primer solution (AP) was added to the sample. They were mixed, collected by brief centrifugation, then the mixture was heated to 70°C for 10 minutes and chilled on ice for at least 1 minute.

The following components were added to this reaction: 2 µl 10x PCR buffer, 2 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 2 µl 0.1 M DTT. Then it was mixed and equilibrated to 42°C for 2-5 minutes, 1µl of Superscript™ II reverse transcriptase was added and the mixture incubated for 50 minutes at 42°C. Finally, to complete this reaction, the sample was incubated at 70°C for 15 minutes and chilled on ice.

The RNA template was removed of the cDNA-RNA hybrid molecule by digestion with 1 µl of RNase H, after first-strand cDNA synthesis to increase the sensitivity of PCR. This reaction was incubated 20 minutes at 37°C and after that stored at –20°C.

2.2.3.2. Isolation of genomic DNA

The method described by Blind and Stafford (1976) was used for the isolation of genomic DNA. Approximately about 100 mg of worms were homogenised with a manual tissue homogeniser, in 1.2 ml of Proteinase K Buffer (100 mM NaCl; 10 mM Tris-HCl pH 8 ; 25 mM EDTA; 0.5% SDS; 0.1 mg/ml Proteinase K) and incubated overnight at 50°C (Thermomixer 5436, Eppendorf) with shaking. The next day an equal volume of phenol/ chloroform/ isoamyl alcohol (Roth, Karlsruhe) was added to each DNA solution to be purified. The samples were mixed manually by invertion and centrifuged for 10 minutes at 4200 rpm (Biofuge^{pico}, Heraeus Holding GmbH). Afterwards the top (aqueous) phase containing the DNA was carefully removed and transferred to a new sterile tube.

To the top aqueous phase 1.5 µl of RNase A (20µg/ml) was added and then incubated at 37°C for 1 hour. Afterwards the samples were reextracted with phenol/chloroform/isoamyl alcohol as in the step described above. The genomic DNA was precipitated with ½ vol of 7.5 M ammonium acetate and 2 vol 100% ethanol and pelleted by centifugation at 13000 rpm for 30 minutes at 4°C (Biofuge^{pico}, Heraeus Holding GmbH). The genomic DNA pellet was washed with 70% ethanol to remove salts and small organic molecules, and again pelleted by centrifugation. Finally, the genomic DNA was resuspended in sterile water and stored at –20°C.

An alternative to this method was realised with the NucleoSpin Tissue Kit ® (Macherey-Nagel, Düren). Approximately 25 mg of worms were placed in a 1.5 ml microcentrifuge tube and homogenized, with a small pestle, in 180 µl of T1 buffer and 25 µl of proteinase K solution. The samples were vortexed to mix and incubated at 56°C (Thermomixer 5436, Eppendorf) overnight until their complete lysis.

The next day 200 µl of B3 buffer was added to each sample and vortexed vigorously to mix and incubated at 70°C (Thermomixer 5436, Eppendorf) for 10 minutes. To each sample 250 µl of ethanol was added and vortexed immediately. Afterwards each sample was applied to the

NucleoSpin Tissue column into a 2 ml collecting tube and centrifuged for 1 minute at 10000 rpm (Table centrifuge 415 C, Eppendorf).

The flowthrough was discarded and 500 µl of BW buffer was added to the column which was then centrifuged for 1 minute at 10000 rpm. These steps were repeated with 600 µl of B5 buffer and then the column was centifuged 3 minutes at 13000 rpm to remove the residual ethanol. The column was placed into new 1.5 ml microcentrifuge tube and 2 x 50µl of prewarmed sterile water (70°C) were added. Finally the column was incubated for 1 minute at room temperature and centrifuged for 1 minute at 13000 rpm to recover the eluted genomic DNA.

2.2.4. DNA electrophoresis

The separation of DNA-fragments was realised by agarose gel-electrophoresis in a Horizontal Mini Submarine Unit (Hoefer He 33, Amersham Pharmacia) as in the method described by Sambrook *et al.* (1989). The gels were prepared in TAE-buffer (0.5 g Agarose; 50 ml TAE-buffer; 5µl Gelstar®) and TAE-buffer was used also as running-buffer.

The DNA samples of 10 µl were mixed with 2 µl of 6x loading-dye and were prepared for electrophoresis at 10 V/cm until the bromophenol blue had migrated into one-half to two-thirds of the length of the gel. To determine the length of the fragments 4 µl of the suitable molecular weight markers (see chapter 1.4) were used. The gel was visualised on a UV light transilluminator (UV-Table TF-M 20x40cm, 312nm; INTAS, Göttingen).

The fragments of interest were cut out of the gel and extracted by centrifugation at 8500 rpm (Biofuge*pico*, Heraeus Holding GmbH), then the fragments were used for cloning.

2.2.5. Enzymatic modification of DNA

The genomic DNA (for the genomic DNA library) was digested with the restriction endonuclease *Eco*R I (Promega, Mannheim). 6.6 µl of 10x buffer and 8.5µl of *Eco*R I (10u/µl) were added to 60 µl (85 µg) of genomic DNA. This reaction was incubated 40 minutes at 37°C (Incubator 3033, GFL, Burgwedel).
The plasmid DNA, obtained of the cloned PCR-fragments, was digested with the same method [2 µl of plasmid DNA; 2 µl of 10x buffer; 0.5µl of *Eco*R I (10u/µl); 15.5 µl sterile water], but the reaction was then incubated 1 hour at 37°C (Incubator 3033, GFL, Burgwedel). 5 µl of each sample were analysed on a 1% agarose gel/Gelstar® and the rest was stored at –20°C until further use. The gels were visualised on a UV light transilluminator (UV-Table TF-M 20x40cm, 312nm; INTAS, Göttingen).

2.2.6. Polymerase Chain Reaction (PCR)

All PCR-Reactions were performed in a PTC-200 DNA Engine™ (MJ Research Inc., Massachusetts, U. S. A) and in Personal Cycler 20 (BIOMETRA®, Göttingen).

2.2.7. Primer and programs

2.2.7.1. HC110-R primer

The oligonucleotide primers for the PCR reactions were designed based on the sequence of the transmembrane receptor HC110-R from *H. contortus* (3539 bp, AC: AJ272270), (Saeger, 2000, Wäring, 2002). All primers were made from the company Invitrogen™ Life tecnologies (Karlsruhe, Germany) (Table.2.1). The melting temperature (Tm) of the primers was calculated with the formula described by Thein and Wallace (1986):
Tm = N x [GC] x 4 + N x [AT] x 2 N = number of base pairs

Several programs and gradients of temperature for each primer pair were used for the amplification of *T. canis* and *A. caninum* PCR-products :

Program 1: 95°C, 3min; 95°C 30 sec; (x)°C, 30sec; 72°C, 30 sec; 72°C 10 min; for 30 cycles. (The [x] is the original annealing temperature of each primer pair).

Program 2: : 95°C, 3min; 95°C 30 sec; 58-68°C, 30sec; 72°C, 30 sec; 72°C 10 min; for 30 cycles.

Program 3: 94°C, 2min; 94°C, 30 sec; 57-68°C, 30 sec; 72°C , 30 sec; 30 cycles; 72°C, 10min, for 30 cycles.

Program 4: 95°C,10 min; 94°C,1min; several temperatures between 55-70°C,1min; 72°C 1min; go to 2 for 30 cycles;72°C 10min.

The degenerate primers for the PCR reactions were designed with the sequence of the transmembrane receptor HC110-R from *H. contortus* (3539 bp, AC: AJ272270), (Saeger, 2000) and with the sequences of orthologous receptors recently identified from *Cooperia oncophora* and *Ostertagia ostertagi* (Welz *et al*., 2005), (Table 2.2). Several programs and gradients of temperature for each primer pair were used for the amplification of *T. canis* PCR-products :

Program 1: 94°C, 10min; 94°C, 30 sec; (X) 30 sec; 72°C 1 min; 72°C, 10 min for 30 cycles. (The [x] is the original annealing temperature of each primer pair).

Program 2: 95°C, 2 min;94°C, 30sec; several temperatures between 60-65°C, 30 sec, 72°C, 1 min; 72°C, 10 min; for 30 cycles.

Table 2.1.: PCR primer pairs used for experiments to amplify an HC110-R orthologous-receptor in *T. canis* and *A. caninum*.

Primer pairs	**Sequences**	**Annealing Temperature (°C)**	**Size of insert (bp) *in H. contortus***	PCR
HC110-F1 HC110-Re1	5' ACCAACATGAGGAATGTATATATT 3' 5' AAAATGACATTCACGACGTCCATC 3'	62/58	300	Tx,Ac cDNA
HC110-F2 HC110-Re2	5' GTGGTTGGTAACGACTTCTTCGT 3' 5' TTCCATGAGGACGTGCCTCTCGT 3'	68/72	305	Tx,Ac cDNA
HC110-F3 HC110-Re3	5' ATGTTCCGCCGAGGGGCTGTGGTCTGAA 3' 5' AAGGGCATTATGTACGACATTCACAAT 3'	86/74	301	Tx,Ac cDNA
HC110-F4 HC110-Re4	5' CGAGCGAAGGAAATGTGGCAG 3' 5' GTAGACTCGGGCACGGTCCAA 3'	66/68	300	Tx,Ac cDNA
HC110-F5 HC110-Re5	5' TATGCGTCTTTTGCGAACATTGGT 3' 5' AGAATTATGCGATTGTAGGGAGCA 3'	68/68	300	Tx,Ac cDNA
HC110-F6 HC110-Re6	5' ACTCATACCGTATGCGCTTGTAGT 3' 5' GCATTCAAACTTTTCTTCTGTCCT 3'	70/66	285	Tx,Ac cDNA
HC110-F7 HC110-Re7	5' GGTATGATCGCTGGTTGCCTCCTTT 3' 5' TACAATGCACATAGTCATCAA 3'	70/56	315	Tx,Ac cDNA
HC110-F8 HC110-Re8	5' TACAGACATTCGAAGTATATCCCCTGT 3' 5' AGAGCTGATACATGAAAGGCCATGCTT 3'	76/78	288	Tx,Ac cDNA
HC110-F9 HC110-Re9	5' GGCTCCCGTGATACCTCTAGGGATCTA 3' 5' GAATGAGGGATAATGGATGGCAACACC 3'	84/80	312	Tx,Ac cDNA
HC110-F10 HC110-Re10	5' GATCTGGATTCCTCCTACCAACCC 3' 5' AACATTTCGCCAATAGTTAGGCGG 3'	74/70	258	Tx,Ac cDNA

Table 2.2. Degenerate PCR primer pairs used to find an orthologous receptor in *T. canis*

Primer pairs	Sequences	Annealing Temp. (°C)	Size of insert (bp)	PCR
HCUNI 1-F1 HCUNI 1-Re1	5' CGAGCGAAGGAAATGTGGCAGAACT3' 5' YGGTGCSACAAAGAAYAARATGAAAAGGTT3'	59	1143	Tx cDNA
HCUNI 2 F2 HCUNI 2 Re2	5' CATATRCCGGTTGCACKYTGTCR 3' 5' ATCATACCRCATTCRAACTTTTCTTCWGTY3'	54.8	187	Tx cDNA

2.2.7.2. Probe primer

The primers for the probes were selected with the program package Oligo 4.1-Primer Analysis Software (National Biosciences Inc., Plymouth,U.S.A) and Lasergene (DNASTAR, Version 5.06), and were designed in two different regions in the sequence of HC110-R, one in the transmembrane region and another with the N-terminal region. A pair of primers was designed based on the fragment of *T. canis* cDNA obtained with the HC110-F/R8 primer by PCR (Table 2.2.).

The following program was used for the PCR amplification of the HCTMR1- F/R and HCNT-2F/R : 95°C, 2 min; 95°C 30 sec; 57°C, 30sec; 72°C, 30 sec; 72°C 10 min; 30 cycles for the steps 2, 3 and 4..

The PCR for the probes TXAP8-F/R and TXBP8-F/R was performed using the following program: Initially 95°C, 10 min; 30 cycles of 95°C 30 sec; 55,5°C, 30sec and 72°C, 30 sec; completed by a final elongation step at 72°C, 10 min.

Table 2.3.: PCR primer used to generate specific probes

Primer pairs	Sequences	Annealing Temp. (°C)	Size of insert (bp)	PCR
HCTMR1-F HCTMR1-R	5' ACGCACCGACAACCTTTTCATTT 3' 5'TAGAGGTATCACGGGACGGAGAGC 3'	57	404	Hc/cDNA
HCNTER1-F HCNTER1-R	5' GGCAGCGAATTCATCTATCC 3' 5' CGCAATTGTTGCGTAGTCAT 3'	60	160	Hc/cDNA
TXAP8-F TXAP8-R	5' CATTCGAAGTATATCCCCTGTAG 3' 5' GCAATATCGTAACGCATCTTCTCTGA 3'	53.9	242	Tx/cDNA

2.2.7.3. ITS Primer

The primers used for the amplification of *A. caninum* und *A. tubaeforme,* first and second ribosomal internal transcribed spacer (ITS) plus the 5.8S gene, were described by Gasser *et al.* (1996) (Table. 4). The following program was used for the PCR amplification: 94°C, 30 sec (denaturation); 55°C, 30 sec (annealing); 72°C, 30 sec (extension) for 30 cycles.

Table 2.4.: ITS Primer pairs.

ITS Primer pairs	Sequences	Annealing Temp. (°C)	Size of insert (bp)	PCR
ITSANCY-F	5' GTAGGTGAACCTGCGGAAGGATCATT 3'	55	800	Genomic DNA
ITSANCY-R	5' TTAGTTTCTTTTCCTCCGCT 3'	55	800	Genomic DNA

2.2.8. Cloning of PCR fragments

The amplified PCR products were cloned into a plasmid vector for sequencing with the TOPO TA Cloning® Kit (Invitrogen). Between 0.5 to 4 µl of each fresh PCR product were mixed with the following reagents: 1µl salt solution;1µl of pCR4 TOPO® vector and sterile water to reach a final volume of 6 µl. The ligation reaction was incubated 30 minutes at room temperature. For transformation approximately 1-2 µl of each ligation reaction was placed into one vial One Shot® TOP10 Chemically Competent *E. coli*, mixed gently and incubated 30 minutes on ice. Then the One Shot® TOP10 cells were shocked with heat (42°C) for 30 seconds and immediately placed on ice for 2 minutes. Following 250 µl of SOC medium was added in each reaction at room temperature and the vials were incubated with shaking 1 hour at 37°C (Incubator 3033, GFL Burgwedel).
Finally 10-50 µl from each transformation were spread on a prewarmed LB-agar plate (50µg/ml Kanamycin) and incubated overnight at 37°C. Single colonies were picked and cultured in 5 ml LB-medium (50µg/ml Kanamycin) overnight at 37°C. These cultures were used for isolation of plasmid DNA. Another alternative method to analyse the transformants was done by PCR with the standard primers T7 (5`GTAATACGACTCACTATAGGGC 3`) and T3 (5`AATTAACCCTCACTAAAGGG3`) or T7 in combination with a unique genespecific primer. Each colony was picked and placed into PCR-cocktail with: 5µl 10x buffer; 1 µl dNTPs; 0.5 µl each primer; 3 µl $MgCl_2$; 9.5 µl Ampuwa water; 0,5 µl Quiagen polymerase.

The amplification was performed in a thermal cycler (PTC-200 DNA Engine, MJ Research Inc., Massachusetts, U.S.A) using the following program: 94°C, 10 min; (94°C, 1 min; 55°C, 1 min; 72°C,1 min) for 30 cycles and completed by a final elongation step at 72°C, 10 min. The sample was analysed on a 1% agarose gel/Gelstar®. The gels were visualised on a UV light transilluminator (UV-Table TF-M 20x40cm, 312nm; INTAS, Göttingen).

2.2.9. Plasmid DNA isolation

2.2.9.1. Mini Plasmid isolation

The plasmid DNA was isolated with the NucleoSpin®Plasmid Isolation Kit (Macherey-Nagel, Düren). This kit is designed for the rapid, small-scale preparation of plasmid DNA by SDS/alkaline lysis (Birboim and Doly, 1979).

The plasmid DNA was isolated out of 5 ml of a saturated *E. coli* LB culture. The saturated culture was pelleted by centrifugation at 4°C for 15 minutes at 3000 rpm; (Heraeus Sepatech, Omnifuge 2. ORS). To the cell pellet 250 µl of A1 buffer was added and resuspended vigorously by vortexing. Then 250 µl of lysis buffer (A2) was added and mixed gently by inverting the tube 6-8 times. This cell suspension was incubated 5 minutes at room temperature.
The resulting cell lysate was neutralised with the addition of 300 µl A3 buffer to create the appropiate conditions for binding of the plasmid DNA to the silica membrane in the nucleospin plasmid column. The cell lysate with the A3 buffer was mixed gently by inverting the tube and centrifuged for 10 minutes at 13000 rpm (Biofuge^{pico}, Heraeus Holding GmbH). Then the supernatant was loaded on a nucleospin column placed into a 2 ml collecting tube and centrifuged 1 minute at full speed.

The flowthrough was discarded and the nucleospin column placed back into the 2 ml collecting tube. 600 µl of A4 buffer was added to wash out the contaminations like salts, metabolites and soluble macromolecular cellular components. The nucleospin column was centrifuged 1 minute at 13000 rpm (Biofuge^{pico}, Heraeus Holding GmbH) to discard the flowthrought, after the silica membrane was completely dried by centrifugation for 2 minutes.

Finally, the nucleospin column was placed in a sterile new 1.5 ml collecting tube and 50 µl sterile water was added. It was incubated 1 minute at room temperature to elute the plasmid DNA of the

column. The column was centrifuged 1 minute at 13000 rpm (Biofuge*pico*, Heraeus Holding GmbH) and the recuperated plasmid DNA was stored at –20°C.

The plasmid DNA was enzymatically digested as described in the chapter 2.2.5 and each sample was analysed on a 1% agarose gel/Gelstar®. The gels were visualised on a UV light transilluminator (UV-Table TF-M 20x40cm, 312nm; INTAS, Göttingen). The concentration was determinated by spectrophotometry.

2.2.9.2. Midi Plasmid isolation

The midi plasmid isolation was performed with the NucleoBond®Plasmid Purification Kit (Macherey-Nagel, Düren). The protocol is based on the SDS/alkaline lysis procedure described by Birboim and Doly (1979).
A single colony was picked and overnight incubated in a 50 ml LB-culture with antibiotic (50µg/ml Kanamycin) as described in chapter (2.2.8). The bacteria were harvested from the saturated LB culture by centrifugation at 3000 rpm for 20 minutes at 4°C (Heraeus Sepatech, Omnifuge 2. ORS).

The pellet of bacterial cells was resuspended in 4 ml of S1 buffer containing 100 µg/ml RNase A. 4 ml of S2 buffer was added and gently mixed by inverting the tube 6-8 times. The mixture was incubated 5 minutes at room temperature. To this mixture 4 ml of pre-cooled S3 buffer was added and immediately mixed by inverting the flask 6-8 times until a suspension containing an off-white flocculate was formed. This suspension was incubated on ice for 5 minutes. During this time a NucleoBond® AX–100 column was equilibrated with 2.5 ml N2 buffer.

To clear the bacterial lysate it was filtered in a prewetted NucleoBond® folded filter and loaded onto a NucleoBond® AX–100 column, allowing the column to empty by gravity flow. The column was washed with 2 x 5 ml of N3 buffer and the flowthrough was discarded.

The column was placed in a new Falcon-tube and the plasmid DNA was eluted by addition of 5 ml of N5 buffer. The plasmid DNA was precipitated with 3.5 ml of 100% isopropanol. This suspension was centrifuged at 13000 rpm for 30 minutes at 4°C (Jouan BR4i, Jouan GmbH) and the supernatant was carefully discarded.

The pellet was washed with 1 ml 70% ethanol and centrifuged at 13000 rpm for 10 minutes at room temperature, the ethanol was discarded and the pellet dried at room temperature 5-10 minutes. Finally the plasmid DNA was resuspended in 50 µl sterile water and stored at –20°C. The concentration and quality of the plasmid DNA was analysed as in the chapter (2.2.1).

2.2.10. Rapid amplification of cDNA ends

The "Rapid Amplification of cDNA Ends"-System (RACE) is a novel method for amplification of nucleic acid-sequences. In essence the RACE protocol generates cDNAs by using PCR to amplify copies of the region between a single point in the transcript and 3`- or 5`- end. To use the RACE, a short stretch of sequence from an exon must be known. From this region, primers oriented in the 3`- and 5`- directions are chosen that will produce overlapping cDNAs when fully extended. Finally the overlapping 3`- and 5`-end RACE products are combined to produce an intact full-length cDNA (Frohman *et al*., 1998 and Loh *et al.*, 1989).

2.2.10.1. Gene-specific PCR-primer

The genespecific-primers were designed of a *T. canis* sequence (233bp), obtained by PCR with the primer pair HC110 F8/Re8, using the program package Lasergene (DNASTAR, Version 5.06). The following characteristics: 23-28 nt long; 50-70 % GC; Tm ≥ 65°C were used for the construction of these primers. The genespecific PCR primers were synthesised by Invitrogen life technologies (Karlsruhe, Germany).

Table 2.5.: *T. canis* genespecific PCR-primer

PCR Primers	Sequences	Anneling Temp. (°C)	GC%
3´GSP1 5´GSP1	5` ATACGCGTTTACCATTGCAAATTCCCTTCAGGGTCTCTT 3` 5` ACACCATCGGGCAATATCGTAACGCATCTTCTCTGAG 3`	73/73	46
3`GSP2 5`GSP2	5` GGAGCGATGGGTCTGGTCTGTCTTCTCGGTGTC 3` 5` CCATCGGGCAATATCGTAACGCATCTTCTCTGAGC 3`	72.9/73.1	50,3

2.2.10.2. 3`-RACE

The 3` RACE-System (BD Biosciences Clontech, U.S.A) uses the poly ($A^{\pm}$) tail of mRNA molecules to start with one special oligo (dT) primer the first-strand cDNA synthesis by reverse transcription. 1-3µl (1µg) of mRNA were mixed with 1µl of 3`-CDS primer A and sterile H_2O was added to a final volume of 5µl. The reaction was incubated at 70°C for 2 minutes and cooled 2 minutes on ice, and the following components were added:

2µl 5x First-Strand Buffer
1µl DTT (20mM)
1µl dNTP Mix (10mM)
1µl BD PowerScript Reverse Transcriptase

The reaction was mixed and incubated at 42°C for 1 1/2 hours in a thermal cycler (PTC-200 DNA Engine, MJ Research Inc., Massachusetts, U. S. A). Finally the first-strand reaction product was diluted with 250µl of Tricine-EDTA Buffer and incubated at 70°C for 7 minutes. The sample can be stored for up to three months at -20°C.

Following to the reverse transcription, the first-strand cDNA (3`-RACE-ready cDNA) was used directly in 3`-RACE PCR reactions with the help of an universal primer A Mix (UPM) and gene-specific primer (GPS1) designed based on the *T. canis* sequence of (233 bp length), which was obtained by PCR with the primer pair HC110 F8/Re8.

The amplification was performed by touchdown PCR (PTC-200 DNA Engine, MJ Research Inc., Massachusetts, U. S. A), each 50µl PCR reaction had the following reagents: 34.5 µl of PCR-Grade water; 5 µl 10X BD Advantage 2 PCR buffer; 1 µl dNTP mix (10mM); 1 µl 50X BD Advantage 2 Polymerase Mix; 5 µl 10X UPM primer and 2.5 µl of 3`RACE-ready cDNA. The following program was used for the amplification:

Program 1: 5 cycles (94°C, 30 sec; 72°C, 3 min); 5 cycles (94°C, 30 sec; 70°C,30 sec; 72°C, 3 min); 25 cycles (94°C, 30 sec; 68°C,30 sec; 72°C, 3 min).

Of each PCR-Reaction 5 µl was analysed on a 1% agarose gel/Gelstar®. The 45 µl rest was stored at –20°C for cloning.

2.2.10.3. 5`- RACE

The 5`- RACE-System (BD Biosciences Clontech, U. S. A) uses also the poly ( $A^{\pm}$) tail of mRNA molecules to start with a special oligo (dT) primer the first-strand cDNA synthesis by reverse transcription. 1-3 µl (1µg) of mRNA were mixed with 1µl of 5`-CDS primer, 1µl BD SMART ll A oligo and sterile H_2O was added to reach a final volume of 5 µl. The reaction was incubated at 70°C for 2 minutes and cooled 2 minutes on ice. The following components were added later:

2µl	5x First-Strand Buffer	1µl	DTT (20mM)
1µl	dNTP Mix (10mM)	1µl	BD PowerScript Reverse Transcriptase

The reaction was mixed and incubated at 42°C for 1 1/2 hours in a thermal cycler (PTC-200 DNA Engine, MJ Research Inc., Massachussetts, U. S. A). Finally, the first-strand reaction product was diluted with 250 µl of Tricine-EDTA Buffer and at 70°C for 7 minutes incubated. The sample can be stored for up to three months at -20°C.

Following to the reverse transcription, the first-strand cDNA (5`-RACE-ready cDNA) was used directly in 5`-RACE PCR reactions with the help of a universal primer A Mix (UPM) and genespecific primer (GPS 2) designed based on the *T. canis* sequence (233 bp lenght),.

The amplification was done by touchdown PCR (PTC-200 DNA Engine, MJ Research Inc., Massachusetts, U.S.A), each 50 µl PCR reaction had the following reagents: 34.5 µl of PCR-Grade water; 5 µl 10X BD Advantage 2 PCR buffer; 1 µl dNTP mix (10mM); 1 µl 50 X BD Advantage 2 Polymerase Mix; 5 µl 10X UPM primer and 2.5 µl of 5`-RACE-ready cDNA.

Program 1: 5 cycles (94°C, 30 sec; 72°C, 3 min); 5 cycles (94°C, 30 sec; 70°C, 30 sec 72°C, 3 min); 25 cycles (94°C, 30 sec; 68°C, 30 sec; 72°C, 3 min).

Of each PCR-Reaction 5 µl were analysed on a 1% agarose gel/gelstar®. The 45 µl rest was stored at –20°C for cloning.

2.2.11. Construction and labelling of cDNA-PCR probes

Several primers were constructed with the sequence of the HC110-R from the parasitic nematode *H. contortus* and a small orthologous sequence previously obtained from *T. canis* by PCR (see chapter 2.2.7.2). These primer were used to generate specific DNA probes by PCR with the PCR DIG Probe Synthesis Kit (Roche, Penzberg) This method was described by Feinberg and Vogelstein (1984). Basically each 50 µl PCR reaction has the following reagents: 36 µl sterile

repurified H_2O, 5 µl PCR-buffer with $MgCl_2$, 5 µl PCR-DIG mix, 0,5 µl of each specific primer, 0.75 µl enzyme mix expand high fidelity and 2 µl Template DNA (*H. contortus* cDNA or *T. canis* cDNA). The amplification was performed in a thermal cycler (PTC-200 DNA Engine, MJ Research Inc., Massachusetts, U.S.A) with the following program: 95°C, 2 min; 30 cycles of 95°C 30 sec; 57°C, 30 sec and 72°C, 30 sec; completed by a final elongation at 72°C 10 min;. Table 2.5. shows the constructed probes, their position and their length.

Of each PCR-Reaction 5 µl were analysed on a 1% agarose gel/Gelstar®. The 45 µl rest were purified with columns of Amicon Microcon® YM-100 (Amicon, U.S.A) by centrifugation at 3000 rpm for 15 min. The following probes were constructed for use in Southern Blot:

Table 2.6.: Probes used to screen the cDNA and genomic DNA libraries. Position and length of the probes.

probe	region	**length (bp)**
Tx-R	Transmenbrane	233
HC-TMR	Transmembrane	404
HC-NT	N-terminus	160

2.2.11.1. Estimation of probe yield

The efficiency of each labeling reaction to determine the optimal amount of probe needed for hybridization was analysed with a direct detection method. In this method, serial dilutions (1 ng/µl- 01 pg/µl) prepared from the DIG-labeled probe was spotted directly on a positively charged nylon membrane to compare with known concentrations of a DIG-labeled control nucleic acid. The serial dilutions of the probe and DNA control spotted on the membrane were

fixed for 5 minutes by UV crosslinking with a UV transilluminator (UV-Table TF-M 20x40cm, 312nm; INTAS, Göttingen). The membrane was stored in a sealed bag at 4°C. The visualization was done using the chemiluminescence detection described in the following chapters.

2.2.12. Southern Blot Analysis

The Southern Blot analysis was performed following the method described by (Southern, 1975). Approximately 10-60 µg of genomic DNA of *T. canis* were digested with the restriction enzyme EcoRI and incubated at 37°C (Incubator 3033, GFL, Burgwedel) for 1 hour. The reaction was run and analysed in a 1% TAE-agarose gel with the appropriate DNA size marker. Transfer of the DNA from an agarose gel onto a positively charged nylon membrane was performed via upward capillary effects with an alkaline buffer. This method was described by Chomczynski (1992).
For the alkaline transfer the gel was pretreated by soaking in denaturation solution 2 x 15 minutes at room temperature and gentle shaking. The denaturation solution was removed and the gel was washed with double destilled water. The water was replaced with neutralization solution and the gel was incubated in this solution for 2 X 15 min at room temperature. Finally, the gel was equilibrated 10 minutes in 20 x SSC buffer.

The blot-transfer was constructed in a “bridge”or “sandwich” of Whatman 3MM paper which had been soaked with 20 X SSC buffer as follows: For supportive reasons a presoaked sponge in 20X SSC Buffer was placed at the bottom. A soaked sheet of Whatman 3MM paper was placed on top. The gel was put onto the paper, supported and covered with a positively charged nylon membrane. To complete the blot assembly a dry sheet of Watman 3MM paper, a stack of papers towels, a glass plate, and a 200-500 g weigh were placed on top. The blot transfer was run overnight. On the next day the membrane was finally washed in 2xSSC for 3 min and the DNA was fixed 5 minutes by UV crosslinking with a transilluminator (UV-Table TF-M 20x40cm,312nm; INTAS, Göttingen). The membranes were stored in a sealed bag until the hybridization at 4°C.

2.2.13. Hybridization

The hybridization was performed following the basic protocol described by Denhardt (1966) and Southern (1975). The membranes were prehybridized for 30 minutes in 5 ml of prewarmed DIG Easy Hyb Buffer (Roche, Penzberg) in a roller bottle (Hybaid HB-OV-BM, GbmH, UK) at 42°C. The hybridization solution was prepared as follows: PCR-labeled probe (2 µl probe/ml hybridation Buffer) was mixed with 50 µl H_2O and then denatured by heating up to 68°C for 10 minutes. Afterwards it was incubated 2 minutes on ice and then the mix was immediately added to prewarmed DIG Easy Hyb Buffer. The prehybridization buffer was replaced with the hybridization solution and incubated overnight at 42°C in the roller bottle.

The next day the hybridization solution was poured out and the membrane washed with Low stringency buffer 2 x 5 minutes, shaking at room temperature. The prehybridized membrane was incubated 2 x 15 minutes in prewarmed High stringency buffer with shaking at 65°C. The high stringency temperature was changed in several experiments to find the correct temperature according to the homology between the PCR-labeled probe and the DNA target. The stringency temperature was changed in a range between 65°- 45°C. The detection of the probe-target hybrids was done by chemiluminescence and visualised by autoradiography.

2.2.14. Chemiluminescence Detection

The chemiluminescence procedure was used to visualise the probe-target hybrids in the Southern Blot with the DIG Wash and Block Buffer Set (Roche Applied Science, Penzberg). The membrane with the fixed probes was washed 2 minutes with DIG-Wash Buffer and incubated for 30 minutes in 1x DIG-Blocking Buffer. Subsequently Antibody-DIG-alkaline phosphatase (1:10.000) in 1x DIG-Blocking Buffer was added and incubated for 30 minutes with shaking.

Finally the membrane was washed 2 x15 minutes with DIG-Washing Buffer and equilibrated 2 minutes in DIG-Detection Buffer.

The positively charged membrane was placed in a plastic bag, chemiluminescent reagent (Ready to use CSPD, Roche, Penzberg) was added and the membrane then incubated for 5 minutes at room temperature and then for 10 minutes at 37°C in an incubator (Incubator 3033, GFL, Burgwedel). The reaction was visualised by autoradiography.

2.2.15. Autoradiography

The autoradiography was done in a dark room to visualise the probe-target hybrids. The membranes in a plastic bag were placed into a metal film-casette (Autoradiography Cassette RPN 1645 35 x 43 cm: KODAK) one sheet of X-ray-film (Lumi-Film Chemiluminescent Detection Film, Roche, Penzberg) was placed over the sealed plastic bag and incubated for 1hour. The X-ray-film was developed (GBX Developer and Replenisher: KODAK, U.S.A.) for 5 minutes approximatly, washed shortly in H_2O for 1 minute and for 5 minutes fixed (GBX Developer and Replenisher: KODAK, U.S.A.) The X-ray-film was washed with water and dried at room temperature.

2.2.16. Construction of cDNA and genomic DNA libraries

2.2.16.1. cDNA library

The *T. canis* cDNA library was constructed using the ZAP Express® cDNA Synthesis Kit (Stratagene, U.S.A). The *A. caninum* cDNA Bank was obtained from the Queensland Institute of Medical Research, Australia . The isolated poly ($A^{\pm}$)-RNA from adult *T. canis* (chapter 2.1.2.3) was used to obtain double-stranded cDNA for the construction of the cDNA library. The

synthesis of the first strand cDNA was prepared with the following reaction: 13.5 µl DEPC-H_2O; 25 µl poly ($A^{\pm}$) -RNA (5 µg); 5 µl 10 x first-strand buffer; 5 µl first-strand methyl nucleotide mixture; 2 µl linker-primer; 1 µl RNAse Block Ribonuclease Inhibitor. This reaction was incubated for10 minutes at room temperature to allow the primer to anneal with the template. 1 µl of StrataScript Reverse Transcriptase was added and this reaction incubated 1 hour at 42°C (Trio-Thermoblock TB1, Biometra, Göttingen). After 1 hour, the reaction was removed and placed on ice. The double strand cDNA was synthetised by adding the following to the reaction: 20 µl 10x second- strand buffer; 6 µl second-strand dNTP mixture; 114 µl DEPC-water; 2 µl Rnase H; 11 µl DNA polymerase I. This reaction was incubated for 2.5 hours at 16°C.

The uneven termini of the double-stranded cDNA were nibbled back by blunting or filled in with cloned *pfu* DNA polymerase. In the next step to the second-strand synthesis reaction the following components were added: 25 µl blunting dNTP mix, 2 µl cloned *pfu* DNA polymerase (2.5 U/µl). This reaction was incubated 30 minutes at 72°C. The reaction was purified and concentrated using the phenol-chloroform method (chapter 2.2.3.2).

The blunt cDNA ends were ligated to *Eco*RI adapters (5`-OH-AATTCGGCACGAGG 3` and 3`-GCCGTGCTCCp-5`). The ligation was done using the following reagents: 9 µl *Eco*R I adapters; 1 µl 10 x ligase buffer; 1 µl 10 mM rATP; 1 µl T4 DNA ligase (4U/ µl) and the bluntended cDNA. The reaction was incubated overnight at 8°C. The next day the ligase was inactivated by incubation at 70°C (Trio-Thermoblock TB1, Biometra, Göttingen). After the ligase inactivation the adapter ends were phosphorylated by adding the following components: 1 µl 10 x ligase-buffer; 2 µl 10 mM rATP; 5 µl sterile water; 2 µl T4 polynucleotide Kinase (5U/ µl). The reaction was incubated 30 minutes at 37°C (Incubator 3033, GFL, Burgwedel) and then heated 30 minutes at 70°C to inactivate the kinase.

Finally the cDNA was digested by 3 µl Xho I (40U/ µl) in a total volume of 53 µl containing 28 µl Xho I buffer for 1.5 hours at 37°C (Incubator 3033, GFL, Burgwedel) to release the *Eco*RI adapter and the residual linker-primer from the 3`- end of the cDNA. The cDNA was precipitated

and concentrated with 5 µl 10 x STE buffer and 100% ethanol and incubated overnight at –20°C. On the following day the precipitation reaction was centrifuged at 13000 rpm for 60 minutes at 4°C and the supernatant was discarded. The pellet was dried and resuspended in 14 µl 1 x STE buffer.

The cDNA was fractionated through a drip column containing Sephacryl-S gel filtration (CromaSpin-1000, Clontech, Heidelberg) to obtain only the large cDNA molecules which were analysed by electrophoresis. This large cDNA with a concentration of 1.5 µg was ligated into ZAP Express® vector with the following reaction: 1 µl ZAP Express® vector (1µg); 0.5 µl 10 x ligation-buffer; 0.5 µl 10 mM rATP; 0.5 µl T4 ligase (2 Weiss U). The reaction was incubated overnight at 4°C. The next day, 4 µl of the ligated cDNA was packed in Gigapack III Gold Packaging Extract (Stratagene, U.S.A). The packaging reaction was incubated for 2 hours at 22°C. To complete the reaction, 500 µl of SM buffer with 20 µl of chloroform were added and mixed. It was then briefly centrifuged to eliminate the cell debris and transferred to a new tube. The cDNA library was stored at 4°C until amplification.

2.2.16.2. Genomic DNA library

The *T. canis* genomic DNA was isolated as described in the chapter 2.2.3.2. Approximately 60µl (85µg) of genomic DNA were digested with the restriction enzyme *Eco*RI (Promega,Mannheim) for 40 minutes at 37°C (Incubator 3033, GFL, Burgwedel). 5 µl of digested genomic DNA were analysed on a 1% agarose gel/Gelstar®. The rest was fractionated through a drip column (ChromaSpin-1000, Clontech, Heidelberg) to eliminate small molecules. The size-selected genomic DNA was concentrated with Amicon Microcon® YM-100 (Amicon, U. S. A) and 2.5 µl (1.5µg) was ligated into ZAP II vector, with the following reaction:1 µl ZAP II vector (1µg); 0.5 µl 10 x ligation buffer; 0.5 µl 10 mM rATP; 0.5 µl T4 ligase (2 Weiss U). The reaction was incubated overnight at 4°C. The next day it was packaged in Gigapack III Gold Packaging Extract (Stratagene (U.S.A). The genomic DNA library was stored at 4°C until amplification.

2.2.16.3. Titer determination and amplification

The cDNA and genomic DNA libraries were amplified and titered with fresh *E. coli* host cells of the XL1-Blue MRF` strain. The host cells were prepared onto LB agar plate containing tetracyclin (12.5 µg/ml). The plate was incubated overnight at 37°C (Incubator 3033, Heraeus, electronic) and then stored at 4°C. Bacteria stock was prepared of this agar plate in 50 ml culture medium (50 ml LB-medium, 500 µl 10 mM $MgSO_4$; 0.2 % Maltose). The culture was incubated overnight at 30°C with shaking at 200 rpm. The next day the bacteria culture was centrifuged and the pellet was resuspended in 10 mM $MgSO_4$ at an OD_{600} of 0.5.

To determine the titer of the packaged ligation product the following components were mixed: 1 µl of the final packaged reaction and 1 µl of a 1:10 dilution the final packaged reaction, both with 200 µl XL1-Blue MRF`cell at an OD_{600} of 0.5. The mix of phage and bacterial was incubated at 37°C for 15 minutes to allow the phage to attach to the cells. In each reaction 3 ml of melted NZY-Top agar were added and plated immediately onto dried prewarmed LB agar plates. The plates were incubated at 37°C for 6-8 hours until the plaques were visible and stored at 4°C. The plaques were counted and the titer was determined using the following formula:

$$\frac{\text{Number of plaques (}pfu\text{) x Dilution Factor}}{\text{Volume of plated (µl)}} \times 1000\ \mu l/ml$$

To amplify the libraries, 10 aliquots of the packaged library suspension containing ~5 x 10^4 *pfu* were mixed each one with 600 µl of XL1-Blue MRF` at an OD_{600} of 0,5, in 10 mM $MgSO_4$ and incubated for 15 minutes at 37°C. 6.5 ml of prewarmed NZY-Top Agar was added to each aliquot of infected bacteria and spreaded evenly onto a freshly poured 150 mm LB-agar plate. The plates were incubated at 37°C for 6-8 hours until the plaques were visible. In the next step the plates were overlayed with 8-10 ml of SM Buffer and incubated overnight at 4°C with gentle rocking. This allows the phages to diffuse into the SM Buffer.

On the next day the bacteriophage suspension from each plate was recovered and pooled into a sterile plastic container. The plates were additionally washed with 2 ml of SM Buffer and pooled. To this suspension chloroform was added to a final concentration of 5% ($^{v}/_{v}$) and incubated for 15 minutes at room temperature. The cell debris was removed by centrifugation for 10 minutes at 500 x *g* and the supernatant transferred into a new container. Finally chloroform was added to a final concentration of 0.3% ($^{v}/_{v}$), the library was stored at 4°C.

2.2.16.4 Blue-white selection and size of inserts

The ratio of the recombinants to nonrecombinants plaques was determined by color selection with IPTG and X-gal. The nonrecombinants plaques were blue and should be < 1x 10^5 pfu/µg of arms, while recombinant plaques were colourless and should be 10-100-fold above the non recombinants. The bacteriophage suspension was plated as described in the chapter 2.2.16.3. with several modifications. The LB-agar plates were streaked with 50 µl of X-gal (250 mg/ml in DMF) and in the NZY-Top agar was added 15 µl of IPTG (in water). The plates were incubated overnight at 37°C and the plaques were analysed and counted. The size of the inserts was analysed by PCR with the following standard primer (Fig. 2.1.):

T3 (5`AATTAACCCTCACTAAAGGG3`)
T7 (5`GTAATACGACTCACTATAGGGC 3`)

Fig. 2.1.: Standard primer used in PCR for analysis of insert size of DNA libraries

Each 50 µl reaction had the following reagents: 38 µl sterile water; 5 µl 10 x buffer; 3 µl $MgCl_2$; 2 µl dNTPs; 0.5 µl primer each; 0.5 µl Quiagen polymerase The amplification was performed in a thermal cycler (PTC-200 DNA Engine, MJ Research Inc., Massachusetts, U.S.A) with the

following program: 95°C, 2 min; (95°C 30 sec; 55°C, 30 sec; 72°C, 30 sec)for 30cycles; and completed by elongation step at 72°C 10 min. Of each PCR-Reaction 5 µl was analysed on a 1% agarose gel/Gelstar®.

2.2.17. Immunoscreening

Approximately, about 5 x 10^4 pfu/plate were combined with 600µl of freshly prepared XL1-Blue MRF` cells (OD_{600} 0,5) and incubated at 37°C for 15 minutes to allow the phages attach to the cells. 6.5 ml of prewarmed NZY-Top Agar was added to each aliquot of infected bacteria and spread evenly onto at least 2 days old 150 mm LB-agar plate. The plates were incubated at 37°C for 6-8 hours until the plaques were visible. Subsecuently the plates were chilled for 2 hours at 4°C to prevent the NZY-Top agar from sticking to the nylon membrane disc.

Over each NZY-Top agar plate a positively charged nylon membrane disc was placed for 2 minutes to allow the transfer of the phage particles to the membrane. The orientation of the membrane was marked with a syringe needle and waterproof pencil. For replicate lift from a single plate a second membrane disc was incubated for 4 minutes to transfer the phages. The membranes were placed side facing up onto a sheet of Whatman 3MM paper to dry briefly. Then the membranes were incubated for 5 minutes in denaturation solution and between each following incubation step placed on a sheet Watman 3MM paper to dry. After the membrane discs were incubated in neutralization solution for 5 minutes.

Finally the discs were incubated in 2 x SSC buffer for 10 minutes and fixed 5 minutes by UV crosslinking using a transilluminator (UV-Table TF-M 20x40cm,312nm; INTAS, Göttingen). The membrane discs were stored in a sealed bag at 4°C until hybridization.

The hybridization and chemiluminescence detection steps were performed as described in the chapters 2.2.13 and 2.2.14 with the following exceptions: the membrane discs were placed in

hybridization bags and incubated in a water bath. Finally the reactions were visualised by autoradiography (chapter 2.2.15). The positive plaques were transferred separately to a sterile tube with 500 µl SM buffer and 20 µl of chloroform. The tube was vortexed and incubated at 4°C until further use.

2.2.18. *In vivo* excision of positive clones

The lambda ZAP II vector is designed to allow simple and efficient in vivo excision and recircularization of any cloned insert contained within the lambda vector, to form a phagemid containing the cloned insert. The *in vivo* excision was performed using the ExAssist Helperphage and the *E. coli* SOLR™. The cultures of XL1-Blue MRF` cells and SOLR cells were handled as described in the chapter 2.2.16.3.

The plaque of interest was transferred to a sterile tube containing 500 µl SM buffer and 20 µl of chloroform. The tube was vortexed and incubated for 1-2 hours at 4°C. Then the following components were combined in a Falcon tube: 200 µl of XL1-Blue MRF` cells (OD_{600} of 1.0); 250 µl of phage stock (>1 x 10^5 phage particles); 1µl of ExAssist helper phage (>1 x 10^6 pfu/µl). The reaction was incubated at 37°C for 15 minutes to allow the phages to attach to the cells. To this reaction 3 ml LB-medium was added and incubated overnight at 37°C. The next day, to lyse the lambda phage particles and the cells, the tube was heated at 65-70°C and centrifuged to pellet the cell debris at 1000 x *g* for 15 minutes (Heraeus Sepatech, Omnifuge 2. ORS).

The supernatant was decanted into a new sterile Falcon tube. This stock contained the excised pBluescript phagemid packaged as filamentous phage particles and was stored at 4°C. To plate the excised phagemids 100 µl of phage supernatant were mixed with 200 µl SOLR cells (OD_{600} of 1.0); and this mixture was incubated 15 minutes at 37°C. Finally 200 µl of the cell mixture were plated on LB-ampicillin agar plates (50µg/µl) and incubated overnight at 37°C.

The colonies appearing on the plate contain the pBluescript double-stranded phagemid with the cloned DNA insert. Single colonies were streaked in a tube with 5 ml LB-medium to do a plasmid isolation as described in the chapter 2.2.9.1. and 2.2.9.2. This plasmid DNA was sent to sequencing.

2.2.19. Analysis of sequences

The plasmid DNA was isolated as described in the chapter 2.2.9. with the Nucleospin® plasmid purification kit (Macherey & Nagel, Düren). The concentration of the plasmid DNA was determined by spectrophotometry (Gene Quantpro RNA/DNA Calculator, Amersham Pharmacia Biotech, Freiburg) and diluted at 0.6 µg for sequencing. The samples were sequenced at the Sequence Laboratories Göttingen GmbH (Seqlab, Göttingen).

The sequences were analysed using the Program Align™ Plus (Sequence Alignment Program Version 4.0, S & E SOFTWARE); Chromas, version 1.45 (Connor McCarthy, Griffith University) and the online databases as the European Molecular Biology Laboratory (EMBL, Heidelberg); GenBank™, Release 93.0 (NCBI, USA) and the „basic local alignment search tool“ (BLAST) (NCBI, USA) (Altschul *et al*., 1990).

3. Results

3.1. *Toxocara canis*

3.1.1. RNA analysis of *T. canis*

The RNA analysis for *Toxocara canis* was performed by using the following methods:

1) Isolation of total RNA
2) Isolation of polyadenylated (A^+)-RNA
3) Quantification of isolated RNA
4) RNA electrophoresis in formaldehyde gel

For each 100 mg of worm between 1.2 – 4.6 µg/µl of total RNA was isolated with the Trizol® reagent (Invitrogen, Karlsruhe) as previously described in the chapter 2.2.1.2 and 2.2.1.3. The quantity of poly (A^+)–RNA obtained with the QuickPrep™ *micro* mRNA purification Kit (Amersham Biosciences) was between 0.2-1.5 µg/µl .

The concentration of RNA-total and mRNA was determined by spectrophotometry (Gene Quantpro RNA/DNA Calculator, Amersham Pharmacia Biotech, Freiburg). The purity ratio was determinated with the standard parameters (see chapter 2.2.1.1). The purity obtained with Trizol® reagent (OD: 1.8) was slightly better than the mRNA isolated with the QuickPrep™ *micro* mRNA purification Kit (OD: 1.3). But a even better quality and purity of mRNA was obtained of the RNA-total isolated with trizol and repurificated with the QuickPrep™ *micro* mRNA purification Kit. The purity obtained of these isolations was of OD: 1.8-1.9. To verify the quality and stability of the RNA, the samples were loaded in a formaldehyde gel electrophoresis (see chapter 2.2.2.1.). The total RNA showed the two typical ribosomal bands of a eukaryotic cells (28S ≈ 4.5 Kb and 18S ≈ 2.0 kb) see Fig. 3.1.

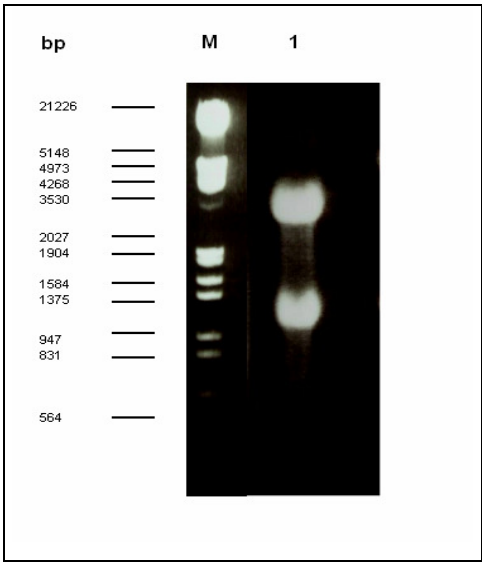


Fig. 3.1.: RNA formaldehyde gel electrophoresis of *T. canis* . M=marker and 1= T. canis RNA.

3.1.2. cDNA synthesis of *T. canis*

Obtained *T. canis* RNA was successfully trancribed into cDNA with the cDNA synthesis Kit of the 3'RACE system for rapid amplification of cDNA (Invitrogen™). The cDNA size distribution was visualised within a range of 0.5-10 kb on a 1% agarose gel/gelstar®

3.1.3. Characterization of PCR products

3.1.3.1. Amplification with HC110-R Primers

To identify a putative orthologous receptor for the anthelmintic PF1022A in *T. canis* ten primer pairs with HC110-R sequence of *H. contortus* (3539 bp) were designed and used to perform

different PCR as described in the chapters 2.2.7.ff. Several programs and gradients of annealing temperatures for each primer pair were used for the amplification of *T. canis* fragments.

To optimise the PCR technique several experiments with differents conditions were performed: annealing temperature, $MgCl_2$ concentration, cDNA quantity. But the PCR had shown multiple products with *T. canis* cDNA (Fig.3.2.). Several products with the approximate length were cut out and cloned for sequencing (Fig.3.3.).

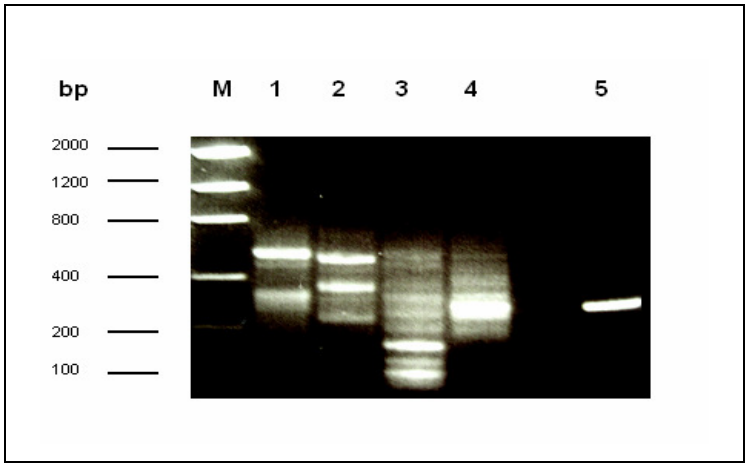


Fig. 3.2.: PCR products with *T. canis* cDNA. M= marker, 1=HC110-F2/Re primer pair,2= HC110-F4/Re primer pair,3= HC110-F6/Re primer pair,4= HC110-F8/Re primer pair and 5= HC control.

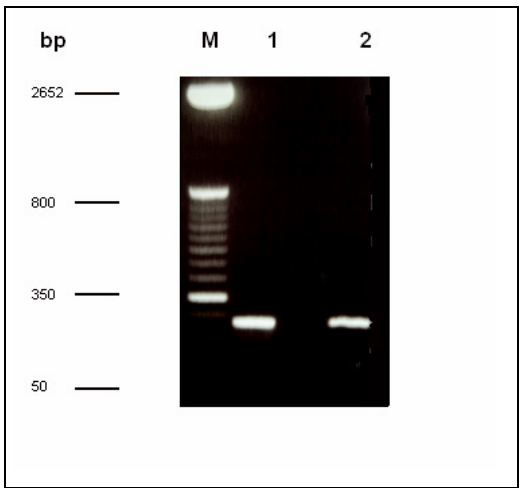


Fig. 3.3.: PCR products of *T. canis* used for cloning and sequencing. M= marker,1= HC110-F8/Re primer pair and 2= HC control.

The sequences were analysed and compared with the sequence of HC110-R with the help of the Program Align™ Plus (Sequence Alignment Program Version 4.0, S & E SOFTWARE) and online data banks like the European Molecular Biology Laboratory (EMBL, Heiddelberg); and GenBank™, Release 93.0 (NCBI, USA).

Of all analysed sequences only one fragment of *T. canis* (233 bp) with similarity to HC110-R was obtained with the primer pair HC110-F8/Re8. It was identified as a putative fragment of the receptor for the PF1022A. The Tx-putative fragment aligned with the cDNA-sequence of HC110-R (AC: AJ272270) from *H. contortus* had a similarity of 100% (Fig. 3.4.). The *T. canis* fragment with similarity is located between the transmembrane domains 5 and 7 (between residues 710-777) of the amino acid sequence of the HC110-R protein. Figure 3.5. shows the possible localisation of the fragment of *T. canis* in the receptor-protein of *H. contortus*.

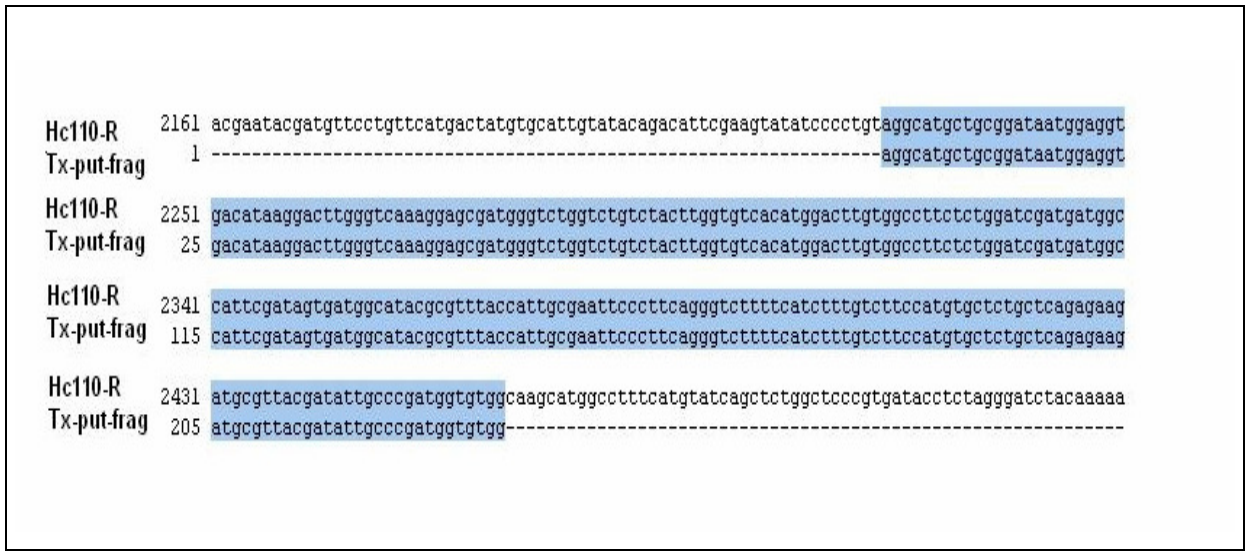


Fig. 3.4.: Alignment of the 233 bp cDNA fragment of *T. canis* and HC110-R from *H. contortus*

The PCR reactions realised with degenerate primers are described in the chapter 2.2.7.1. The amplified products only showed multiple bands. Each single band with the aproximate length was cut out and cloned (see chapter 2.2.8.). The sequences were analysed with help of the programs described in chapter 2.2.19, but of the sequences analysed none could be identified as a part of the *T. canis* putative orthologous receptor for the PF1022A.

Fig. 3.5 Alignment of amino acid sequence of HC110-R from *H. contortus* and the 233 bp cDNA fragment of *T. canis*

3.1.3.2. RACE Amplification

The "Rapid Amplification of cDNA Ends"-System (RACE) was used for amplification of full-length cDNA sequence of the *T. canis* putative fragment to obtain the complete orthologous receptor. Gene specific primers were designed of the known sequence of the Tx-putative fragment (233bp) (see chapter 2.2.10.1.l.).

The RACE-PCR has the same problem of muliple bands like the normal PCR amplification with the HC110-R primers (Fig.3.6.). In the RACE-PCR amplification it was not possible to obtain a single band. The PCR standard programs were modified but the multiple bands did not increase. Each single band with the aproximate length was cut out and cloned (see chapter 2.2.8.). The sequences were analysed with help of the programs described in chapter 2.2.19.

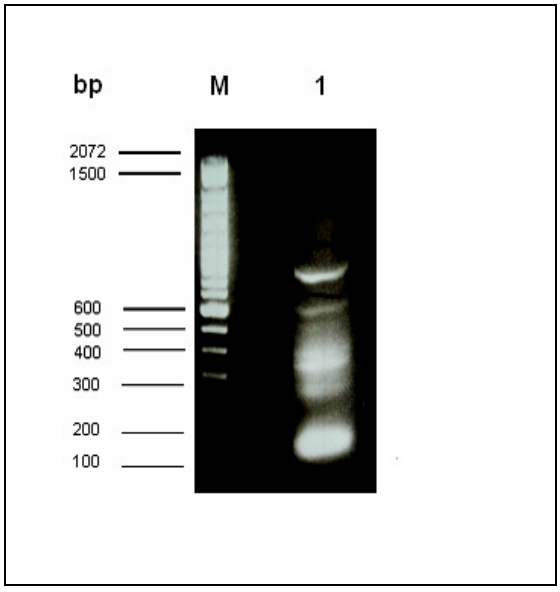
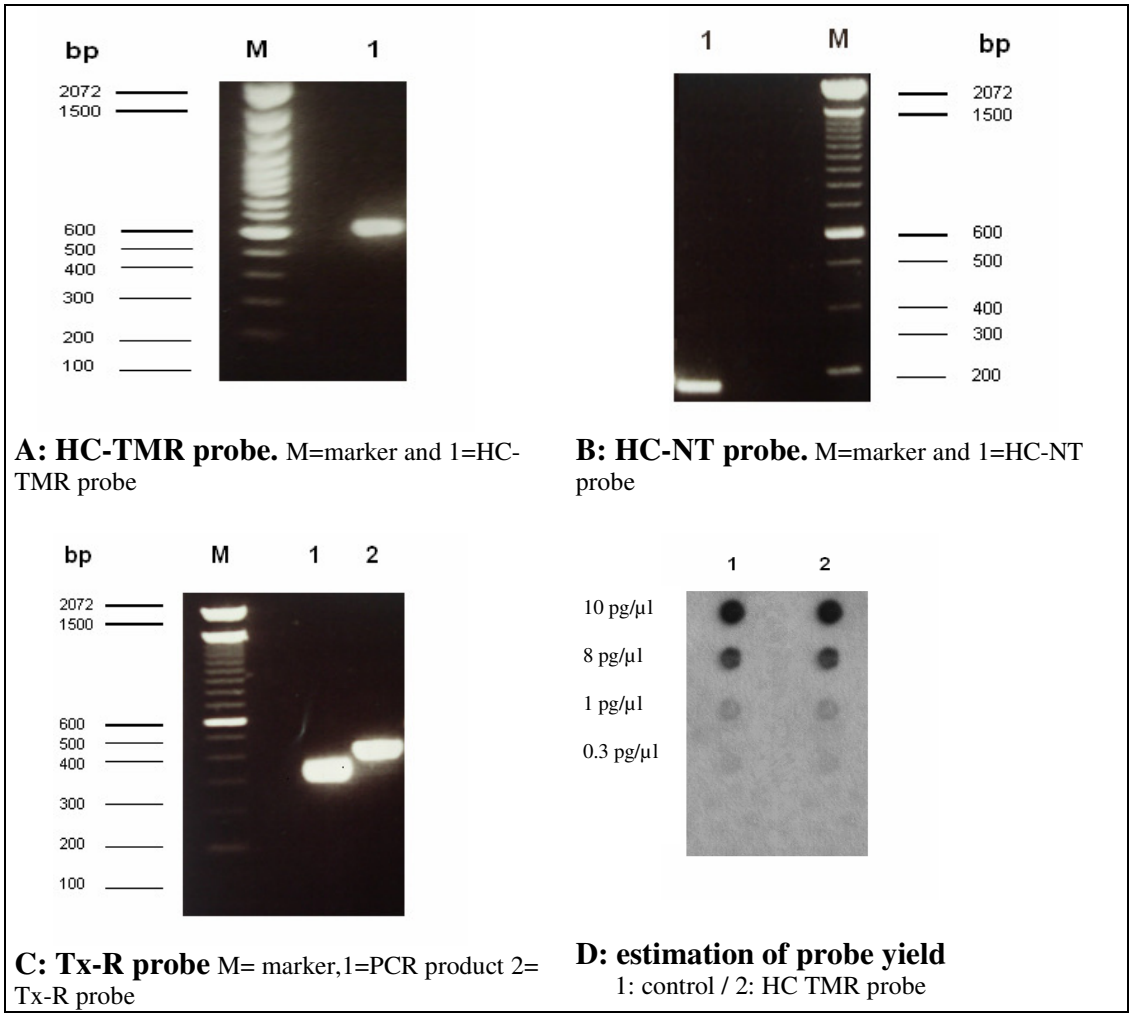


Fig. 3.6.: Example of RACE PCR products of *T. canis* .M= marker and 1=*T. canis* RACE product.

Of the sequences analysed none could be identified as a part of the *T. canis* putative orthologous receptor for the PF1022A.

3.1.4. Probe construction and analysis of probe yield

Three probes were constructed by PCR with the PCR DIG Probe Synthesis Kit (Roche, Penzberg) as described in the chapter 2.7.1.2. and analysed on a 1% agarose gel/Gelstar® (Fig. 3.7. A-C). The efficacy of each labeling reaction was analysed with a direct detection method (see chapters 2.2.11.1 ff) to determine the optimal amount of probe to add in the hybridization and to determine the detection sensitivity. The probes can be used to detect a signal up to 0.3 pg/µl (Fig. 3.7. D).



A: HC-TMR probe. M=marker and 1=HC-TMR probe

B: HC-NT probe. M=marker and 1=HC-NT probe

C: Tx-R probe M= marker,1=PCR product 2= Tx-R probe

D: estimation of probe yield
1: control / 2: HC TMR probe

Fig. 3.7.: A-C: Examples of electrophoresis gels with the three different probes
D: Estimation of probe yield

3.1.5. Southern Blot analysis

The quantity of genomic DNA obtained with the classical method by Blin and Stafford (1976) was slightly higher than the quantity obtained with the NucleoSpin Tissue Kit ® (Macherey-Nagel, Düren). Of 100 mg of worm with the classical method were isolated between 0.5 – 2.2 µg/µl of genomic DNA and with the NucleoSpin were isolated between 0.5–1.3 µg/µl. But purity and quality (OD, size) was better with the classical method. The genomic DNA of *T. canis* was digested with the restriction enzyme *Eco*RI and incubated at 37°C (Incubator 3033, Heraeus electronic).

To determine the best DNA digestion time, which the restriction enzyme *Eco*RI, the genomic DNA was incubated with the following incubation times: 5 min, 10 min, 20 min, 30 min, 40 min, 60 min, 90 min (Fig. 3.8.). The best incubation time to digest the genomic DNA was 40 min.

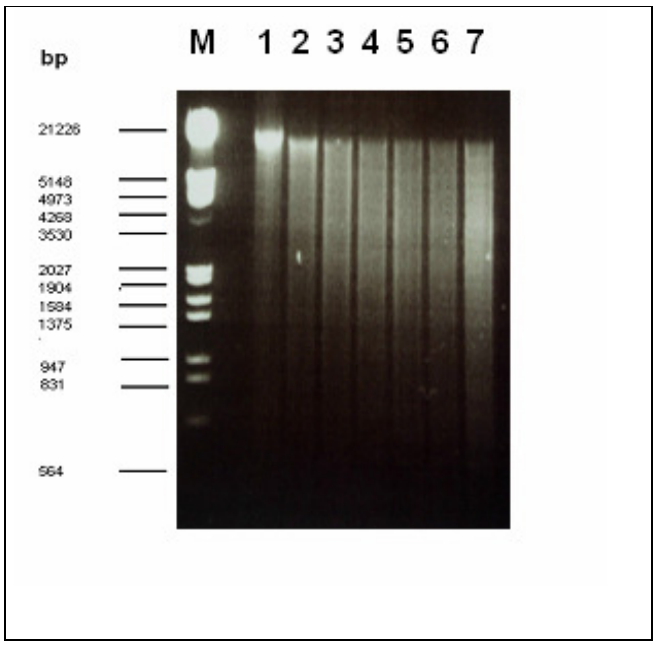


Fig. 3.8.: Incubation time of *T. canis* genomic DNA digestion. M= marker, 1=5 min, 2= 10 min, 3= 20 min, 4= 30 min, 5= 40 min, 6= 60 min and 7= 90 min.

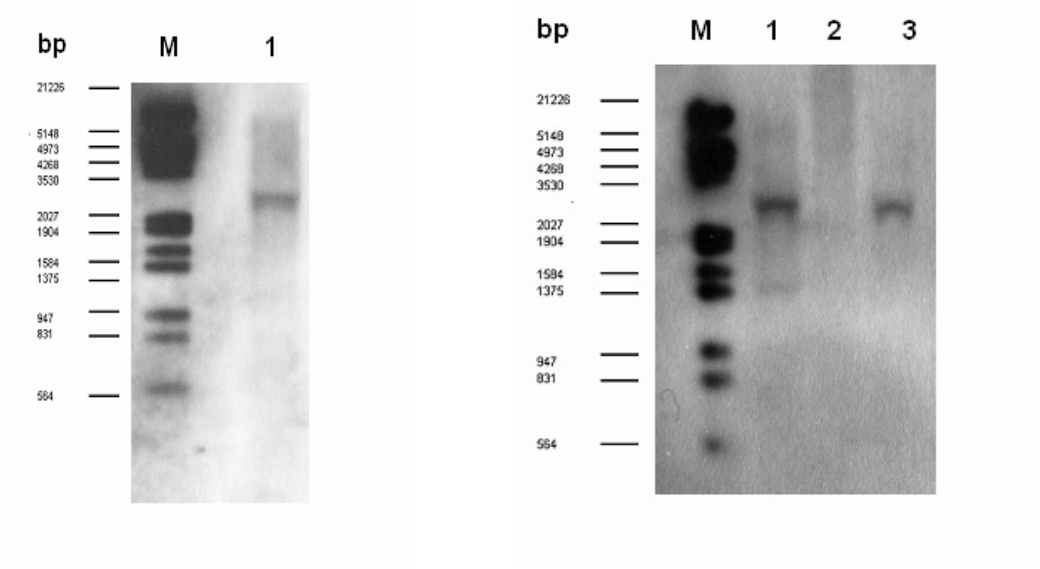
The genomic DNA was run and analysed in a 1% TAE-agarose gel with an appropriate DNA size marker. The genomic DNA was transferred from an agarose gel onto a positively charged nylon membrane via upward capillary with an alkaline buffer system (see chapter 2.2.12.). The hybridization was performed with the three probes constructed in the chapter 3.1.4. (Tx-R, HC-TMR; HC-NT) with a temperature of 42°C.

The hybridization was performed with different high stringency temperature to find the optimal temperature according to the homology between the PCR-labeled probe and the DNA target. The genomic DNA of *H. contortus* was used as control. To find the suitable stringency temperature it was changed between 65°- 45°C.

The optimal temperature for the high stringency wash step, with the genomic DNA of *H. contortus* for all three probes was 65°C. The temperature to detect probe-target hybrids using the genomic DNA of *T. canis* and *A. caninum* was 45°C with the three probes HC-TMR, HC-NT and Tx-R. The detection was realised by chemiluminescence as described in chapter 2.2.14. and the probe-target hybrid was visualised by autoradiography (see chapter 2.2.15.).

The Southern Blot method was successfully used to localise the Tx–putative fragment sequence and the probes sequences of the HC110-R receptor in the genomic DNA of *T. canis*.

In the Southern Blots, performed with the HC-TMR probe, we detected a strong band of a length approximately 2.5 kb for *H. contortus* and *T. canis*. The Southern Blots with the HC-TMR probe also showed a light band of a length 1.3 Kb (Fig.3.9. A+B).

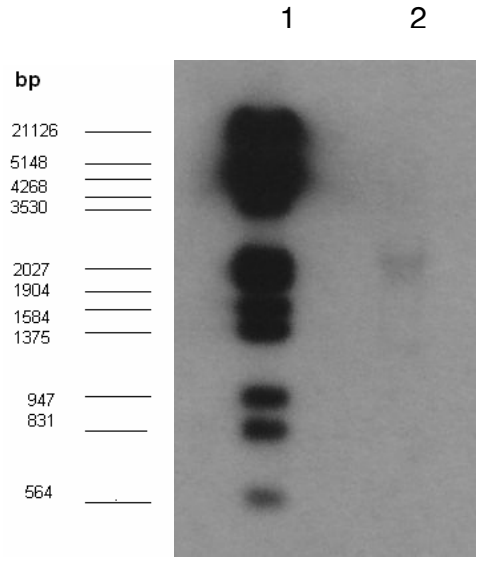


A: Southern Blot of *H. contortus* with the HC-TMR probe, M= marker / 1= Hc genomic DNA

B. Southern Blot of *T. canis* genomic DNA with: M= marker, 1= HC-TMR probe, (2= empty) 3= HC-NT probe,

Fig. 3.9.: A+B: Examples of Southern Blot membranes from the two parasites *H. contortus* and *T. canis* with different tested probes

The Southern Blots performed with the Tx-R probe showed a signal of ca. 2.5 kb length (Fig.3.10.). The results of the Southern Blot with the three probes demonstrated that the number of copies of this gene might be very small in the genome of this parasites.



C: Southern Blot of *T. canis* with Tx-R probe 1= marker and 2= *T. canis* genomic DNA

Fig. 3.10.: Example of a Southern Blot membrane obtained from *T. canis* with the Tx-R probe

3.1.6. Characterization of cDNA and genomic DNA libraries

The genomic DNA library and the cDNA library of *T. canis* were constructed as described in the chapter 2.2.16.

The amplified cDNA library of *T. canis* has a primary titer of 1 x 10^6 pfu/ml. The porcentage of nonrecombinants plaques was 3%. This porcentage was determined by blue-white selection as described in the chapter 2.2.16.4.

The quality of a cDNA bank is determined by the length of the inserts. The used lambda ZAP® II vector has six unique cloning sites that will acommodate DNA inserts from 0 to 10 Kb in length.

To determine the length of the cloned cDNA fragments, 50 plaques were picked and characterized by PCR. The constructed *T. canis* cDNA library showed only small inserts (100-500 bp), therefore it is non representative and they can not be used to find the desired sequence.

The constructed genomic DNA of *T. canis* has a titer of 1.8 x 10^7 and the porcentage of nonrecombinants plaques was 1%. The inserts lengths were characterized from 50 clones by PCR and the results are shown in fig. 3.11.

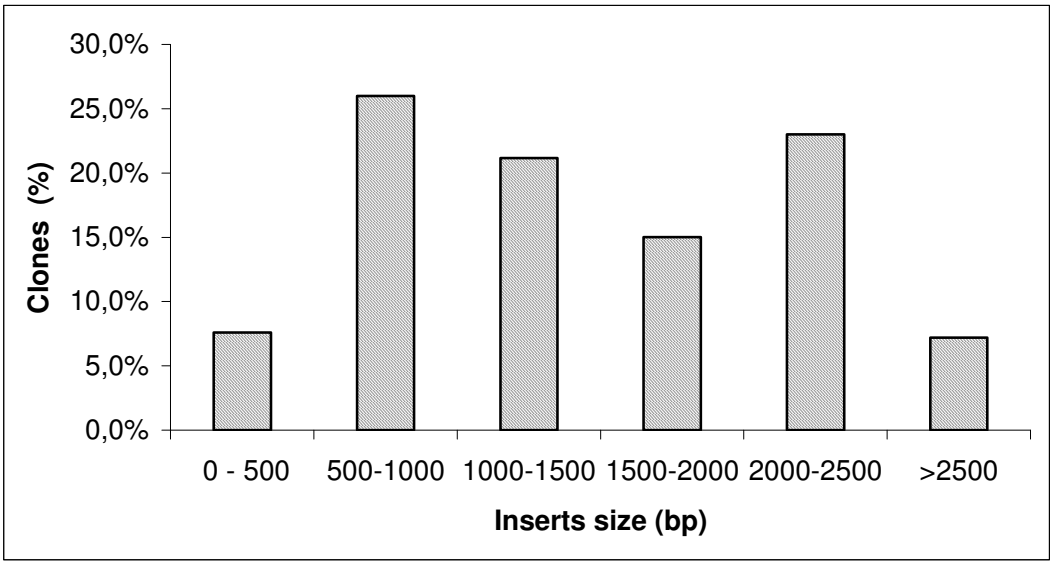


Fig. 3.11.: Insert size of the clones obtained from the genomic DNA of *T. canis*

X= Inserts size in bp Y= Clones (%)

3.1.7. Immunoscreening of *T. canis*

The genomic DNA library of *T. canis* was used to find the desired clones by immunosreening. The hybridization was performed using cDNA probes used in the Southern Blot (see chapter 2.2.12.). The positive clones were detected by chemiluminescence and visualised by autoradiography (see chapters 2.2.14. and 2.2.15.).

For the library an approximate total of 1,3 x 10^7 plaques was screened. In the first screening of the genomic DNA library 32 plaques were detected as positive. To eliminate the "false" positive clones, these plaques were used for a second screening. In this second screening only 13 plaques were detected as positve, the rest of plaques were possibly false positive results. The positive plaques were used to realise an *in vivo* excision (see chapter 2.2.18.) and later sent to sequencing. Unfortunately the obtained sequences of these plaques did not encode the desired sequence.

Examples of the sequences are shown for comparison in table 3.1.

Table 3.1.: Examples of sequences obtained by immunoscreening of the genomic library of *T. canis* * Length of the fragment with similarity from the complete sequence

Clones of *T. canis*	**Sequence**	**Length* (bp)**	**Similarity (%)**
Tx-01	*Ascaris lumbricoides* 5.8S ribosomal RNA and 28S ribosomal partial sequence	231	100
Tx-02	*C. elegans* YAC Y47D3b	21	87
Tx-03	*T. canis* small subunit ribosomal RNA	253	100
Tx-04	*C. elegans* acetylcholine receptor	21	100

3.2. *Ancylostoma caninum*

3.2.1. RNA analysis of *A. caninum*

The RNA analysis for *Ancylostoma caninum* was performed by using following methods:

1. Isolation of total RNA
2. Isolation of polyadenylated (A^+)-RNA
3. Quantification of isolated RNA
4. RNA electrophoresis in formaldehyde gel

For each 100 mg of worm between 1.2 – 3.2 µg/µl of total RNA was isolated with the Trizol® reagent (Invitrogen, Karlsruhe) as previously described in the chapter 2.2.1.2 and 2.2.1.3. The quantity of poly (A^+)–RNA obtained with the QuickPrep™ *micro* mRNA purification Kit (Amersham Biosciences) was between 0.5-1.7 µg/µl **.**

The concentration of total RNA and mRNA was determined by spectrophotometry (Gene Quantpro RNA/DNA Calculator, Amersham Pharmacia Biotech, Freiburg). The purity ratio was determinated with the standard parameters (see chapter 2.2.1.1). The purity obtained with Trizol® reagent (OD: 1.8) was significantly better than the mRNA isolated with the QuickPrep™ *micro* mRNA purification Kit (OD: 1.2). But a better quality and purity of mRNA was obtained of the total RNA isolated with Trizol® and repurificated with the QuickPrep™ *micro* mRNA purification Kit The purity obtained of these isolations was of OD: 1.8-1.9. To verify the quality and stability of the RNA, the samples were loaded in a formaldehyde gel electrophoresis (see chapter 2.2.2.1.). The total RNA showed the two typical ribosomal bands of a eukaryotic cells (28S ≈ 4.5 kb and 18S ≈ 2.0 kb) see Fig. 3.12.

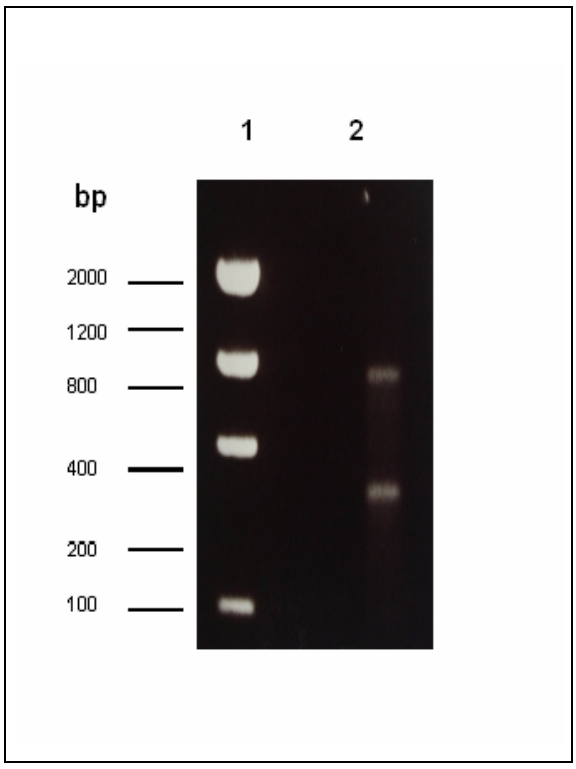


Fig. 3.12.: RNA gel electrophoresis of *A. caninum* 1= marker and 2= *A. caninum* RNA

3.2.2. cDNA synthesis of *A. caninum*

Obtained RNA of *T. canis* was successfully trancribed into cDNA with the cDNA synthesis Kit of the 3'RACE system for rapid amplification of cDNA (Invitrogen^{TM}).

3.2.3. Characterization of PCR products

For *A. caninum* only the amplification with the HC110-R primer (see Table 2.1) was performed. The amplified products only showed multiple bands, so that no further sequencing was performed.

3.2.4. Probe construction and analysis of probe yield

For *A. caninum* the same probes were used as for *T. canis.* For detailed description see chapter 3.1.4.

3.2.5. Southern Blot analysis

As seen for *T. canis*, the quantity of genomic DNA obtained with the classical method by Blin and Stafford (1976) was slightly higher than the quantity obtained with the NucleoSpin Tissue Kit ® (Macherey-Nagel, Düren). Of 100 mg of worm with the classical method between 0.5 – 3.2 µg/µl and with the NucleoSpin between 0.5 – 1.2 µg/µl were isolated. But purity and quality was better with the classic method. The genomic DNA of *A. caninum* was digested with the restriction enzyme *EcoRI* and icubated at 37°C (Incubator 3033, Heraeus electronic).
The incubation time for digestion of the genomic DNA was determined for *A. caninum* the same way than for *T. canis* and 40 min were also found to be the best incubation time for digestion of the genomic DNA.

The genomic DNA was run and analysed the same way as described for *T. canis* in chapter 3.1.5.

The hybridization was performed with different high stringency temperature to find the optimal temperature according to the homology between the PCR-labeled probe and the DNA target. The genomic DNA of *H. contortus* was used as control. To find the suitable stringency temperature it was changed between 65°- 45°C.

The optimal temperature for the high stringency wash step, after the hybridization of probe-target hybrids for the three probes with the genomic DNA *H. contortus* as control, was 65°C. The temperature to detect probe-target hybrids using the genomic DNA of *A. caninum* was 45°C with

the two probes HC-TMR and HC-NT. The detection was realised by chemiluminescence as described in chapter 2.2.14 and the probe-target hybrid was visualised by autoradiography (see chapter 2.2.15.).

In the Southern Blots, performed with the HC-TMR probe, a strong band of a length of 2.5 kb was detected for *A. caninum*. The Southern Blots with the HC-NT probe also showed a band in the same position (Fig. 3.13.).

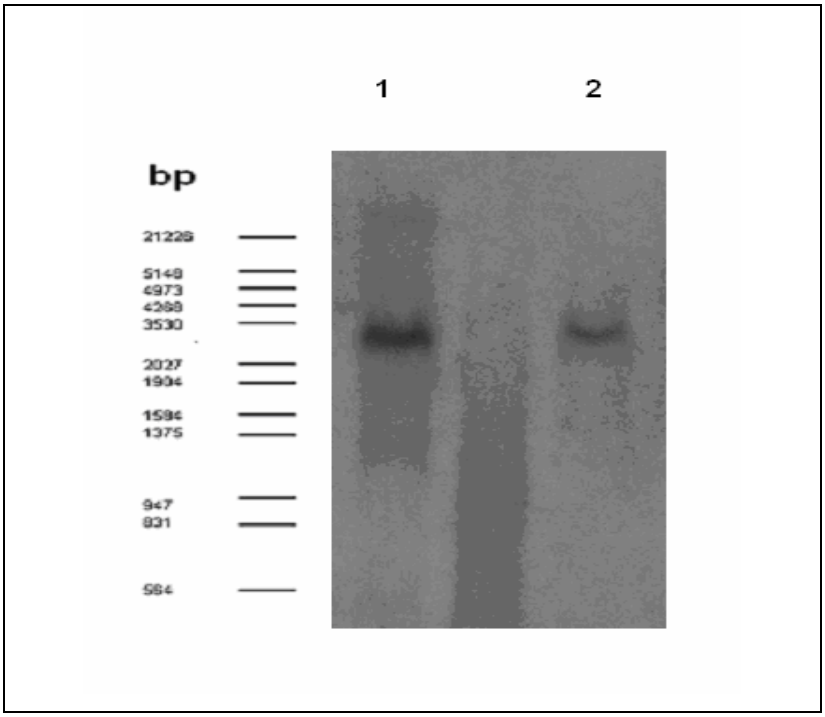


Fig. 3.13.: Southern Blot of *A. caninum* with 1= HC-MTR probe and 2= HC-NT probe

3.2.6. Characterization of the cDNA library

The cDNA library of *A. caninum* was obtained from the Queensland Institute of Medical Research, Australia and was constructed also with the method described in the chapter 2.2.16.1

The amplified cDNA library of *A. caninum* had a titer of 4.4 x 10^7 and the porcentage of nonrecombinants was 2%. The quality of a cDNA bank is determined by the length of the insert. The used lambda ZAP® II vector has six unique cloning sites that will acommodate DNA inserts from 0 to 10 kb in length. To determine the length of the cloned cDNA fragments, 50 plaques were picked and characterized by PCR. The constructed *A. caninum* cDNA library showed inserts between (0 - 2.500 bp). Therefore the library was representative and could possibly be used to find the desired sequence. The inserts size distribution is summarized in the Fig. 3.14.

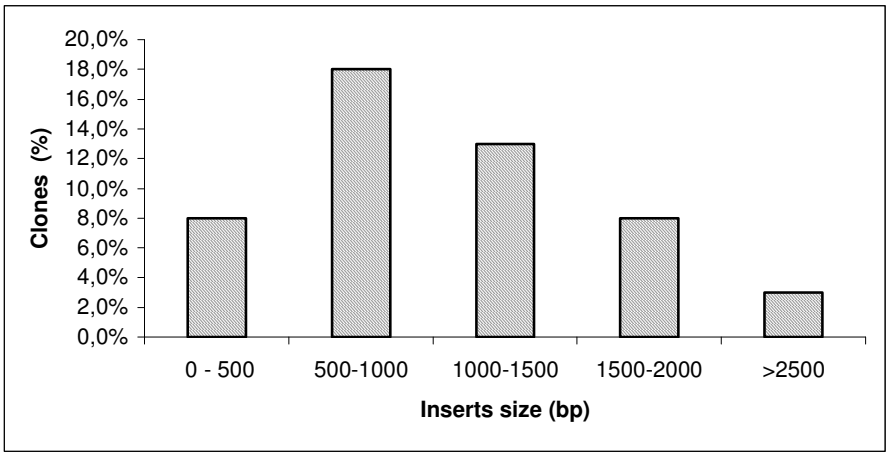


Fig. 3.14.: Insert size of the clones obtained from the genomic DNA of *A. caninum*

3.2.7. Immunoscreening

For the library an approximate total of 1.3 x 10^7 plaques were screened. The cDNA library of *A. caninum* was screened with the previously described procedure for the genomic DNA library of *T. canis*. A total of 14 plaques was detected as positive in the first screening and only 6 plaques were detected as positive in the second screening. The positive plaques were used to realise an *in vivo* excision (see chapter 2.2.18.) and later send to sequencing. But unfortunately the obtained sequences of these plaques did not encode the desired sequence. Table 3.2 shows examples of the sequences obtained from the clones of *A. caninum*.

Table 3.2: Examples of sequences obtained by immunoscreening of the cDNA library of *A. caninum* *Length of the fragment with similarity from the complete sequence

Clones of *A. caninum*	Sequence	Length*(bp)	Similarity (%)
Ac-01	*Uncinaria stenocephala* small subunit ribosomal RNA	738	93
Ac-02	*Mus musculus* similar to flightless I homolog	124	84
Ac-03	*Homo sapiens* BAC clone RP11-404P12	24	100
Ac-04	*C. elegans* cosmid Y39A3CL	453	95

3.2.8. Internal transcribed spacer (ITS)

A total of 30 ITS sequences of *A. caninum* and 30 sequences of *A. tubaeforme* were obtained fromsingle worm DNA and analyzed with help of the programs described in the chapter 2.2.19. No sequence variation was found within individual species (=intraspecific variation) in both parasites. The alignments showed a similarity of 100% between the 30 sequences of *A. caninum* and the same was shown in the 30 sequences of *A. tubaeforme.* The level of ITS sequence variation (=interspecific variation) observed among *A. caninum* and *A. tubaeforme* is only 3% (see Fig. 3.15.)

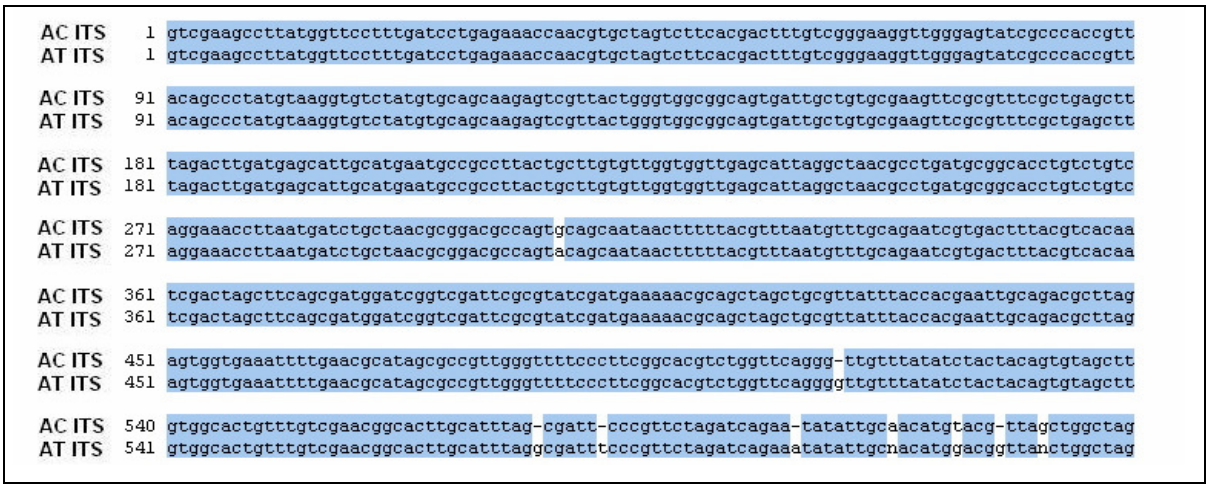


Fig. 3.15.: Alignment of the ITS sequence obtained from *A. caninum* and *A. tubaeforme*

4. Discussion

The aim of this work was the identification and characterisation of a putative orthologue of the HC110-R receptor of *T. canis* and *A. caninum*. In *H. contortus* the HC110-R receptor has been identified as a target protein for the binding of PF1022A, a new anthelmintic belonging to the class of cyclooctadepsipeptides.

In a PCR, based on primers generated from HC110-R, a 233 bp long fragment could be amplified for *T. canis*. This fragment showed a high similarity with the transmembrane domain of HC110-R in *H. contortus*. The *T. canis* fragment with similarity is located between the transmembrane domains 5 and 7(between residues 710-777) of the amino acid sequence of the HC110-R protein. The amplification of other fragments with degenerate primers and further experiments with the amplification of the cDNA ends (RACE), however, were not yet possible.

In the Southern Blot, bands in the genomic DNA of *T. canis* and *A. caninum*, digested with *Eco*RI, were detected. The Southern Blot was performed with the help of several DNA-probes, generated from a known sequence of the HC110-R. For both isolates as well as for *H. contortus*, the bands were found with a length of approximately 2.5 kb. The identification of these bands in the Southern Blot proves that in both organisms, *T. canis* and *A. caninum*, prospective Hc110-R orthologues with sequence similarity high enough to bind to receptor-specific probe, exist.
In the following, possibilities are discussed why the several molecular methods performed did not lead to the detection of the complete sequence for the putative orthologue of HC110-R in *T. canis* and *A. caninum*.

Test material

The worms used were stored at -80° C. Therefore a possible degradation of the RNA cannot be excluded. The mRNA of an organism is digested by RNases. The half-life of mRNA can be only

several minutes (Knippers, 2001). In the living organism there is a balance between transcription and disassembling. If the organism dies the homoeostasis cannot be maintained. The preservation of mRNA is only possible if special RNase deactivation-buffer and extremely low temperatures (-80°C) are used (Fedorcsak and Ehrenberg, 1996). Even in these low temperature regions, the degradation of mRNA can occur (Eigner *et al*., 1961).

Another problem could be the variable expression of the receptor. In *C. elegans* it is reported that the HC110-R orthologous receptor "latrophilin-like protein1" is mainly expressed in the pharynx and the somatic body musculature (Mee *et al*., 2004). Nothing is known if this expression changes within the life cycle of this organism, however given a lack of expression in the adult worm seems to be unlikely.

RNA Isolation and cDNA Synthesis

The putative mRNA of the receptor is believed to be approximately 3.5 kb in length. This is a relatively long molecule and likely to be damaged in the purification process. Therefore in the subsequent cDNA synthesis, fewer molecules are available which possess the necessary poly-A tail. Furthermore incomplete transcription is possible if the Reverse-Transcriptase stops the process prematurely. In the obtained cDNA, the occurrence of the HC110-R orthologue can be less than the occurrence of other smaller molecules. This is a problem if the gene which is used is not abundant and not expressed in high quantity.

PCR

Primers

The primer pair HC110-F1-10 and HC110-Re1-10 were designed on the HC110-R sequence, which has been used previously to describe the genomic DNA of *H. contortus* (Wäring, 2002). The two primers HC110-F8 / Re8 were successfully used for the amplification of the 233 kb long

fragment in *T. canis*. These primers are localised in the transmembrane domain. This area is relatively well conserved, a similarity of the orthologous receptors is expected (Saeger, 2000). All other primer pairs used, which are also based on the HC110-R sequence, did not generate any positive results. The fact, that none of the tested primers within the transmembrane domain annealed was unexpected because of the high similarity of the fragments of *T. canis* to HC110-R.

Degenerate Primers

The degenerate primers which were generated based on the sequences from HC110-R and the putative orthologues in *C. oncophora* and *O. ostertagi* (Welz *et al*., 2005), also did not lead to clear amplification products. Degenerate primers are designed based on similarities of known sequences. During the primer synthesis, different nucleotides are incorporated in positions where different sequences show different nucleotides. Afterwards in the PCR-reaction there are several primers for the different known sequences available. There is therefore a chance that the unknown sequence of the tested organism, which might only be a little different, could be detected and amplified.

To obtain enough primer with the same sequence in the primer-mix for the PCR, only a few positions can be degenerate in every primer. The degenerate positions should be close to the 5'-end to allow a successful annealing at the 3'-end. This is important for the function of the polymerase.

With degenerate primers, it is possible to amplify orthologous sequences of different organisms, but the danger of obtaining non-specific products is relatively high. The typical picture seen on the agarose gel after a PCR reaction with degenerate primers shows multiple bands. The superposition of non-specific and specific bands is also a problem. After the cloning, it is possible that clones with the expected sequence might be present, but cannot be detected due to low concentration.

RACE PCR

For RACE PCR experiments, a gene-specific primer and a universal primer, binding either to the 3' or 5'-end of the analysed DNA, are used. Therefore the possibility of obtaining non-specific products is quite high. To avoid these non-specific products, very long gene-specific primers with a high melting temperature are used. The known fragment of *T. canis* is very short and therefore does not offer a lot of possibilities for designing gene-specific primer. In the RACE-experiments only non-specific products were amplified.

Southern Blot

In the Southern Blot, genomic DNA of *T. canis* and *A. caninum* was analysed with HC110-R probes and compared with *H. contortus.*

In all three attempts, a band of approximately 2.5 kb could be isolated. To preserve the bands, the stringency of conditions had to be kept as low as possible. The following screening of the genomic and cDNA libraries has been performed under low stringent conditions. The probes detected several clones as positive, which were found non-specific in the following sequencing process. The non-specific annealing is likely to be due to the low stringent conditions. The exact localisation and the picking of the plaques after the Southern Blot were problematic. Therefore it is also possible, that a positive clone gave the signal, but a non-specific clone was amplified. In order to obtain a higher quantity and to ease the isolation, the whole area surrounding a positive clone was again amplified on new plates. It cannot be excluded, that this put pressure on the selection of smaller inserts, which again lead to the identification of non-specific sequences. In the screening of the genomic DNA library of *T. canis* could be detected introns and they can interfere in the detection of positive clones. E.g. the probes anneal on a short exon-segment but in the process only an intron-segment, which shows no homologues, has been sequenced and a positive clone can remain undetected.

With none of the used molecular methods a complete sequence of the putative HC110-R receptor could be identified in *T. canis* and *A. caninum*. But it was possible to prove, that the sequence is present in the genetic material of *T. canis* and *A. caninum* by Southern Blot.
In all methods the same problems and difficulties had to be worked with; to allow a non-specific annealing or amplification up to the stage, that the sequences are recognised even if they are definitely different from the known HC110-R sequence.

On the other hand the false positive identification of non-specific products had to be avoided, but unfortunately this was not possible in all cases. A possible explanation could be to relatively high divergence of the sequences. The three analysed species all belong to different families: *H. contortus* to the family of Trichostrongylidae, *T. canis* to the family of Ascarididae and *A. caninum* to the family of Ancylostomatidae (Bowman *et al*, 2003). However, the high similarity of the 233 kb long fragment of *T. canis* with the HC110-R of *H. contortus* implicates, that a high divergence of the sequences is unlikely. A possible explanation could be also the possibility of contamination with *H. contortus* cDNA in any step of the used methods.

It would have been of special interest to perform a Southern Blot with cDNA of the tested organisms, in order to localise the size of a band and to evaluate the possible size of the receptor-cDNA. If the length of this band would be definitely smaller or bigger than in *H. contortus*, it would submit the possibility, that the receptor is smaller / bigger. This difference could then explain the difficulties in amplification and detection of the sequences.

Internal transcribed spacers (ITS)

The ribosomal internal transcribed spacers (ITS 1 and ITS 2) sequences are the markers most commonly used to discriminate among nematode species (Powers *et al*., 1997; Gasser and Newton, 2000; Blouin, 2002).

With ITS-PCR we obtained a 800 bp product, that contains the ITS 1; ITS 2 and the 5,8S gene, for *T. canis* and *A. caninum*. Nevertheless, for both parasites, sequence variations within individual species (=intraspecific variation) were not found. This is also described in previous studies, where the level of ITS sequence variation observed among individuals of the same species is about the same as the level observed among ITS repeats within individuals, typically ≤ 1% in *H. contortus* and *H. placei* (Stevenson *et al.,* 1995); bursate nematodes (Gasser *et al*.,1998) and Ascarididae (Nadler *et al.,* 2000).

We found a level of ITS sequence variation (=interspecific variation) of 3% among *A. caninum* and *A. tubeaforme*. Chilton *et al.* (1999) reported a interspecific variation between 0.8 - 3.2% for these two species.

The molecular techniques used in this study were shown to be a useful tool for the identification of an orthologous receptor of the HC110-R in *T. canis* and *A. caninum.* But the results indicate that for further screening of libraries of *T. canis* and *A. caninum*, probes have to be optimised in order to be more specific for the targeted species and the design of a high number of degenerate primers could help to find the rest of sequence of those receptors with PCR.

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6. Erklärung

Hiermit erkläre ich, dass ich vorliegende Doktorarbeit selbständig verfasst habe und keine anderen, als die angegebenen Quellen und Hilfsmittel verwendet habe.

Die vorliegende Dissertation ist nicht schon als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden.

Hannover, den 25. Juli 2006

-Edith Ulate-

7. Lebenslauf

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