Characterization of the antigenicity of cuticular collagens of nematodes

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IMPRIMATUR POUR LA THESE

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Summary

Lymphatic filariasis is caused by thread-like, parasitic filarial nematodes. This disease is a major social and economic burden in the tropics and sub-tropics of Asia, Africa, the Western Pacific and parts of the Americas, covering over120 million people in more than 80 countries. In tropical and subtropical areas where the disease is well established, the incidence of infection is continuing to increase. This is primarily due to the rapid and unplanned growth of cities, which creates numerous breeding sites for the mosquitoes that transmit the disease. Today, lymphatic filariasis is a significant cause of acute and chronic illness in both urban and rural areas, affecting people of all ages and both sexes, particularly the poor and vulnerable. The *World Health Report* in 1995 identified lymphatic filariasis as the second leading cause of permanent and long-term disability worldwide. Earlier methods were insufficient to control the disease in many countries. However, during the past decade, significant research progress has led to an increased understanding of the severity and impact of the disease, the development of new diagnostic tools and most importantly, new treatments and control methods. The development of practical, feasible control measures that can be applied on a community-wide basis and integrated within existing public health activities, has renewed hope for eliminating the disease and reducing suffering.

Presently, the best form of individual prevention is avoidance of contact with the infective stage of the parasites. In the case of lymphatic filariasis, this would be avoidance of mosquito bites by using repellents, mosquito nets and protective clothing. This clearly indicates the need of further methods to control nematode infections and it seems evident that the best preventive protection would be a specific immunoprophylactic vaccine.

The overall aim of the present thesis is to identify, proteinaceous molecules that are involved in the interaction between the parasite and the host during the infection process. Nematodes constitute one of the most abundant groups of animals. Their filiform morphology in combination with the fact they are entirely covered by a layer of proteins that forms the cuticle, have enabled them to colonise many environments. Several species are parasitic in insects, vertebrates or plants. This extra-cellular matrix consists of several layers that protect the nematode from the adverse conditions of the environment and at the same time forms the site of important exchanges. In parasitic species it constitutes an interface between the nematode and its host, i.e. a barrier between the worm and the vertebrate's immune response. The interactions occurring between the parasite and its host are critical for the success of the infection, during which the cuticle plays a key role in controlling the interactions. As more than 80% of the cuticle is composed of specific nematode collagens, the present thesis is focused on these collagen proteins. Although the cuticular collagens are usually not directly exposed to the host immune system, some collagen fragments from the old cuticle are present in the blood during each moult, making them accessible to the immune system of the host cells. For this reason, it is interesting to study the structure of cuticular collagens. A better understanding of the molecular structure of this surface is essential, since the successful establishment of the parasite within its host depends on the composition of the cuticle and on the interactions with the host.

The free-living nematode worm *Caenorhabditis elegans*, the intestinal parasite *Ascaris suum* and the filarial parasite *Brugia pahangi* were used to study the protein profiles of the different cuticular collagens. The comparison of these different protein profiles confirms that the major structural components of the cuticular matrix consist of a set of collagen proteins (>80%) of various molecular weights, ranging from 30 kDa to more than 200 kDa. The exact molecular weights of the collagen differed between the three different nematodes, but the predominant ones were usually between 90-120 kDa. The collagen molecules were found to be present in the form of monomers, dimers and trimers. The monomers are the 30 kDa polypeptides, the dimers are represented by the group of polypeptide between 50-70 kDa and the trimers by the group of 90-120 kDa polypeptides. The exact molecular weight of the collagen species

depends on the transcription of the corresponding gene, as well as the relative amounts of cross-linking between the proteins and the location of the cross-links in the polypeptide chains.

Studies on cuticular collagen proteins revealed that they are strongly immunogenic. This was confirmed by the finding that collagen molecules of nematode parasites are early-recognized parasite components in infections of laboratory animals. Moreover, it was found that the monomeric form is less immunogenic than the trimeric form, indicating that the immunogenicity depends on the structure and molecular weight of the collagens.

No specificity was observed for antibodies raised against cuticular collagen when tested by immunoblot analysis of different cuticular collagen proteins from different species of nematodes. However, one antibody was specific to its immunogen. This antibody (a-*Bpcol-1*) was obtained by immunizing mice with a recombinant cuticular collagen protein of *B. pahangi*. This recombinant protein was obtained from a yeast-based expression system of the *Bpcol-1* gene of *B. pahangi*. Immunoblot analysis of this antibody shows no cross-reaction when tested on *A. suum* and *C. elegans* cuticular collagens.

This study demonstrated that even if cuticular collagen of nematodes are quite similar in their structure and show high levels of homology in their sequences, they posses specific immunogenic properties.

Résumé

La filariose lymphatique est causée par un ver parasite de la famille des nématodes. Cette maladie est la cause de l'un des fardeaux économique et social les plus important dans la partie tropical et subtropical de l'Asie, de l'Afrique, de l'Est du Pacific et d'une partie des Amériques, touchant plus de 120 millions de personnes dans plus de 80 pays. Dans les régions tropicales et subtropicales, où la maladie est bien établie, le risque d'infection est sans cesse croissant. Ceci est en majorité du à l'accroissement démographique non planifié des villes, ce qui crée de nombreux lieu de reproduction pour les moustiques qui sont les vecteurs de cette maladie. Aujourd'hui, la filariose lymphatique est une cause importante de maladie aiguë et chronique autant dans les régions urbaines que rurales, touchant des populations de tout âge et des deux sexes, particulièrement les populations pauvres et vulnérables. Le rapport mondial de la santé de 1995 a identifié la filariose lymphatique comme étant la seconde cause d'infirmité permanente ou à long terme dans le monde. Dans de nombreux pays, les premières méthodes furent insuffisantes pour contrôler la maladie. Cependant, au cours des dix dernières années, des recherches importantes ont permis d'accroître nos connaissances sur la gravité et l'impact de cette maladie, et ont également permis de développer de nouveaux outils de diagnostiques et de nouvelles méthodes de contrôle et de traitement. Ces mesures de contrôle ont redonné l'espoir de pouvoir éliminer la maladie et réduire le nombre de victimes souffrant de cette maladie.

Actuellement, la meilleure forme de prévention individuelle est l'absence de contact avec la forme infective du parasite. Dans le cas de la filariose lymphatique, ceci correspond à l'absence de contact avec les piqûres de moustiques en utilisant des répulsifs, des moustiquaires et des habits qui protègent. Ceci démontre clairement le besoin de développer de nouvelles méthodes pour contrôler les infections par des nématodes et il semble évident que ce soit dans une protection préventive comme un vaccin immunoprophylactique.

Le but principal de ce présent travail, est d'identifier la nature protéique de molécules impliquées dans l'interaction hôte-parasite qui intervient lors d'une infection par un nématode. Les nématodes comptent parmi les animaux les plus nombreux. Leur morphologie filiforme ainsi que le fait qu'ils sont entièrement recouverts d'une couche de protéines qui forme la cuticule sont des conditions qui leur a permis de coloniser de nombreux milieux. De nombreuses espèces sont parasites d'insectes, de plantes ou de vertébrés. Cette matrice extracellulaire est constituée de plusieurs couches et protège l'animal des agressions de son environnement. Elle est le siège d'importants échanges et dans le cas des espèces parasites, elle constitue une interface entre le nématode et son hôte, une barrière entre le système immunitaire du vertébré et le ver. Les interactions entre le parasite et son hôte jouent un rôle primordial dans la réussite de l'infection, et la cuticule est à coup sur le siège de telles interactions. Etant donné que plus de 80% de la cuticule est composée de collagènes spécifiques aux nématodes, ce présent travail traitera des protéines collagènes. Bien que les collagènes cuticulaires ne soient habituellement pas directement exposés au système immunitaire de l'hôte, des fragments de collagènes de l'ancienne cuticule peuvent néanmoins être présents dans le sang au moment de chaque mue, et sont de ce fait accessibles au système immunitaire des cellules. Pour cette raison, il est intéressant de travailler sur les collagènes cuticulaires. Une meilleure compréhension de la structure moléculaire de cette surface du nématode est indispensable pour mieux comprendre la réussite de l'établissement de ce parasite à l'intérieur de son hôte. Cet établissement dépend de la composition de la cuticule et de son interaction avec son hôte.

Le nématode libre *Caenorhabditis elegans,* le parasite intestinal *Ascaris suum* et le parasite filaire *Brugia pahangi* ont été utilisé pour étudier les profils protéiques de ces différents collagènes cuticulaires. La comparaison de leur différents profils protéiques a permis de confirmer que le composant principal de la cuticule est fait de protéines collagènes (>80%) de

différents poids moléculaires allant de 30 kDa à plus de 200 kDa. Le poids moléculaire exact de ces protéines collagènes varie parmi ces trois nématodes, mais le plus présent se situe aux alentours de 90-120 kDa. Ces molécules de collagène sont présentes sous forme de monomères, de dimères et de trimères. La forme monomère correspond au polypeptide de taille 30 kDa, la forme dimère est représentée par le groupe de polypeptides compris entre 50 et 70 kDa et la forme trimère par le groupe de polypeptides compris entre 90 et 120 kDa. Le poids moléculaire exact de ces collagènes dépend d'une part du gène collagène qui est exprimé et d'autre part du nombre de réactions croisées entre les protéines et de la localisation de ces réactions croisées dans la chaîne polypeptidique.

Des études sur les protéines de collagènes cuticulaires ont démontré que ces protéines sont fortement immunogènes. Ceci est confirmé par le fait que des molécules collagènes de nématodes parasites sont très vite reconnues en tant que composants du parasite lors d'infection d'animaux de laboratoire. De plus il a été démontré que la forme monomère est moins immunogène que la forme trimère, ce qui indique que l'immuogénicité dépend de la structure et du poids moléculaire des collagènes.

Aucune spécificité n'a été observée chez des anticorps dirigés contre des collagènes cuticulaires lorsque ceux-ci ont été utilisés pour des analyses sur immunoblot contre différentes protéines de collagènes cuticulaires de différentes espèces de nématodes. Cependant, un anticorps a démontré une spécificité. Cet anticorps (a-*Bpcol-1*) a été obtenu en immunisant des souris avec une protéine recombinante de collagène cuticulaire de *B. pahangi.* Cette protéine recombinante a été obtenue par l'expression du gène *Bpcol-1* présent chez *B. pahangi.* Cette expression à été réalisée à l'aide d'un système d'expression de levures. Les analyses d'immunoblot de ces anticorps n'ont démontré aucune réaction croisée lorsque ces anticorps ont été testés sur des collagènes cuticulaires d'*A. suum* et de *C. elegans*.

Cette étude a démontré que malgré le fait que les collagènes cuticulaires de nématodes sont relativement similaires au niveau de leur structure et démontrent une forte homologie au niveau de leur séquence, ils possèdent des propriétés immunologiques spécifiques.

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Abbreviations

Bp	Base pair
BSA	Bovine Serum Albumine
°C	Celsius grade
DEC	Diethylcarbamycin
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediamino-tetraacetic acid
EtOH	Ethanol
g	Gramm(and all its subunits)
IgG	Immunoglubuline G
HMW	High Molecular Weight
IPTG	Isopropyl-ß-D-thiogalactoside
Kb	Kilo base
kDa	Kilo Dalton
W	Watt (and all its subunits)
L	Liter (and all its subunits)
LB	Luria-Bertani medium
m	Meter (and all its subunits)
M	Molar (and all its subunits)
mA	MilliAmper (10^{-3} A)
2-ME	2-mercapto-ethanol
MeOH	Methanol
min	Minute
OD	Optical Density
PAGE	Polyacrylamide gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMSF	Phenylmethansulfonyl fluorid
rpm	Rotations per minutes
SDS	Sodium dodecylsulfate
sec	Second
Taq	*Thermos aquaticus*
TBE	Tris Buffered EDTA
TBS	Tris Buffered Saline
TE	Tris EDTA
Tris	Tris-(hydroxymethyl)-aminomethane
TTBS	Tween TBS
V	Volt(and all its subunits)
X-gal	5-bromo-4-chloro-3-indolyl-ß-galacto-pyranoside

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CHAPTER 1 : INTRODUCTION

1 Nematodes

The nematodes, or 'round worms', are a part of a large assemblage of relatively simple structure with a widespread distribution, their cylindrical non-segmented bodies distinguishing them easily from other helminthes. Nematodes are among the most abundant animals on earth. They occur in fresh water, in the sea and in the soils, and are among the most common parasites of plants and animals. Some 49% of the 80'000 described nematode species are parasites of plants, invertebrates and vertebrates. Most of the free-living nematodes are microscopic, as well as many of the parasitic species invading the body fluids such like the blood or the lymph channels. The species, which live in the intestine, are generally larger, while some in tissue habitats grow sometimes to relatively enormous lengths (more than 8,5 m for *Placentonema gigantissimum* a parasite that invades the placenta of the whale) (Smyth, 1994).

Nematodes are elongated, tapered at both ends, bilaterally symmetrical, and possess a pseudocoel, that is, a body cavity derived from the embryonic blastocoel. The pseudocoel exists however under a large variety of different shapes. The digestive system is complete, with a mouth at the anterior extremity and an anus near the posterior tip. The lumen of the pharynx is characteristically triradiate. The body is covered with a noncellular cuticle that is secreted by an underlying hypodermis and is shed four times during ontogeny. The muscles of the body wall are only one layer thick and are distinguished by being longitudinally arranged with no separate circular layer. The excretory system consists of lateral canals or ventral canals or eventually both of them, which open near the anterior end through a ventral excretory pore. With the exception of some sensory endings of modified cilia, neither usual cilia nor flagella are present, even in the male gamete. Most nematodes are dioeciously and show considerable sexual dimorphism: the females are usually larger, and the tail of the male is more curled. Some species are hermaphroditic, and others are parthenogenetic. The female reproductive system opens through a ventral genital pore; the male system opens into a cloaca, together with the digestive system (Smyth, 1994).

Anatomically, nematodes are comprised of two concentric tubes, the intestine being the internal, and the cuticle, hypodermis, muscle layers and nervous system, the external. These two concentric structures are separated by the pseudocoelomic space, which in the adult worm also contains the gonad (Fig.1) (Politz, and Philipp, 1992).

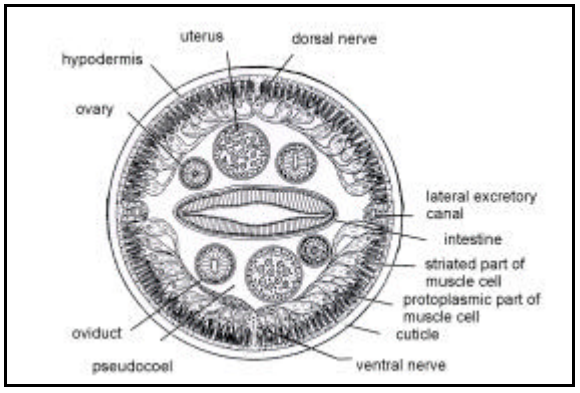


Fig. 1: *Ascaris lumbricoides.* Diagrammatic transverse section showing general morphology (After Brown, 1950) (Wood, 1988).

An agreed classification of nematodes has been difficult to devise and agreement is still not universal. However the separation into two classes presented by Maggenti (1981) is accepted. Nematodes have been traditionally divided broadly into the *Aphasmidea* (*Adenophorea*) and

the *Phasmidea* (*Secernentea*), depending on whether caudal sense organs, the phasmids, are present or not (Table 1).

Class	Order	Family	Genus, Species
Adenophorea	Trichocephalida	Trichuridae	*Trichuris sp.*
		Trichinellidae	*Trichinella sp.*
	Mermithida	Mermithidae	
Secernentea	**Rhabditida**	**Rhabditidae**	*Rhabdias bufonis*
			Caenorhabditis elegans (free-living soil nematode)
	Strongylida	Strongyloididae	*Strongyloides stercoralis*
		Ancylostomatidae	*Ancylostoma sp., Necator sp.*
		Trichostrongylidae	*Trichostrongylus sp.,*
			Haeamonchus contortus
		Dyctiocaulidae	*Dictyocaulus sp.*
	Ascaridida	**Ascarididae**	***Ascaris suum***
		Toxocarididae	*Toxocara sp.*
		Oxyuridea	*Enterobius vermicularis*
	Spirurida	**Filariidea**	Filarial worm
			Acanthocheilonema vitae
			Brugia pahangi, *Onchocerca volvulus*
	Camallarida	Dracunculoidea	*Dracunculus medinesis*
	Diplogasterida		Parasites of insects
	Aphelenchida		Parasites of plants and insects
	Tylenchida		Parasites of plants and insects

Table 1: Classification of nematodes. Only a selection of some orders and species of nematode is presented here (Mehlhorn, 1988).

The process of growth in size of nematodes is accompanied by moults. In general, there are four moults and the nematode formed as the fifth stage is the adult, although some growth in size may follow. The stages in the postembryonic growth of nematodes are usually referred to as “larvae” although according to some authors “juveniles” is a more suitable term for such stages.

After the first moult, larvae are termed second-stage larvae; after the second, third-stage, and so on. The stage at which the larvae are capable of infecting the host is termed the infective stage.

The degree of development of the egg when laid varies considerably with species. In some, the eggs are laid in an unsegmented condition (e.g *Ascaris* and *Trichuris*), while in others early segmentation has commenced (e.g. hookworms). Still others (e.g. *Syphacia*) may reach a stage with a coiled larva visible and in the ovoviviparous species and the so-called viviparous species, fully developed larvae are released by the females (e.g. Dracunculus) (Smyth, 1994).

The biology and the life cycles of three nematode species are described in more detail because they were studied in this thesis more closely.

2 Caenorhabditis elegans

Caenorhabditis elegans is a small free-living soil nematode found commonly in many parts of the world. It feeds primarily on bacteria and reproduces under optimal conditions with a life cycle of about 3 days (Fig.3). The two sexes, hermaphrodites and males, are each about 1 mm in length but differ in appearance as adult. Hermaphrodites produce both oocytes and sperms and can reproduce by self-fertilization. Males, which arise spontaneously at low frequency, can fertilize hermaphrodites; hermaphrodites cannot fertilize each other. The male tail has specialized neurons, muscles and hypodermal structures for mating that gives it quite a different appearance from that of the hermaphrodite (Fig. 2). The male tail is fan-shaped with 18 sensory rays. At the base of the tail are two spicules, which are inserted into the hermaphrodite vulva during copulation to aid in transfer of sperm (Wood, 1988).

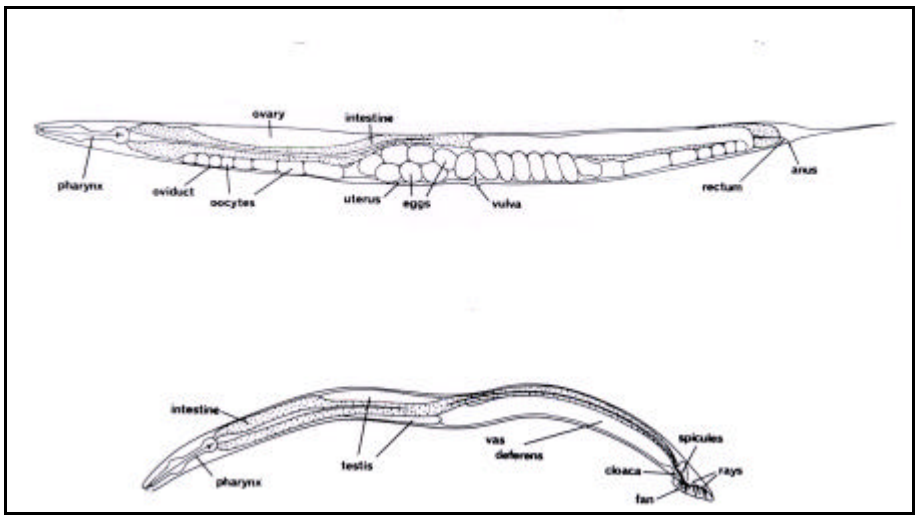


Fig. 2: Photomicrographs showing major anatomical features of *C. elegans* adult hermaphrodite (above) and male (below) (Wood, 1988).

C. elegans reflects the typical nematodes body plan described by Wharton (1986). *C. elegans* is easily maintained in the laboratory, where it can be grown on Agar plates or in liquid culture with *Escherichia coli* as a food source. The key attributes of *C. elegans*, as an experimental system for biological studies, are their simplicity, transparency, ease of cultivation in the laboratory, short life cycle, suitability for genetic analysis, and small genome size. *C. elegans* is used as an "*in vitro*" model of nematodes due to the similarity of the cuticle's structure compared to other nematodes.

Like other nematodes, *C. elegans* sheds and replaces its cuticle at each of four postembryonic molts. Juvenile worms hatch and develop through four stages (larval stages) punctuated by molts. The mature adult emerging from the fourth molt is fertile during 4 days and then lives for an additional 10-15 days. Cuticle proteins are synthesized at high rates during the molts and at lower rates between molts (Wood, 1988).

In response to adverse conditions such as lack of food, the L2 larva molts into a specialized stage called the dauer larva, which is a developmental alternative to the L3. The dauer larva is developmentally arrested and can survive for several times the normal life span in the absence of food. Dauer larvae are more resistant to noxious chemicals than are the other stages

(Cassada, and Russell, 1975; Klass, and Hirsh, 1976). When it encounters suitable environmental conditions, the dauer larva resumes development by molting into an L4 (Fig.3) (Wood, 1988).

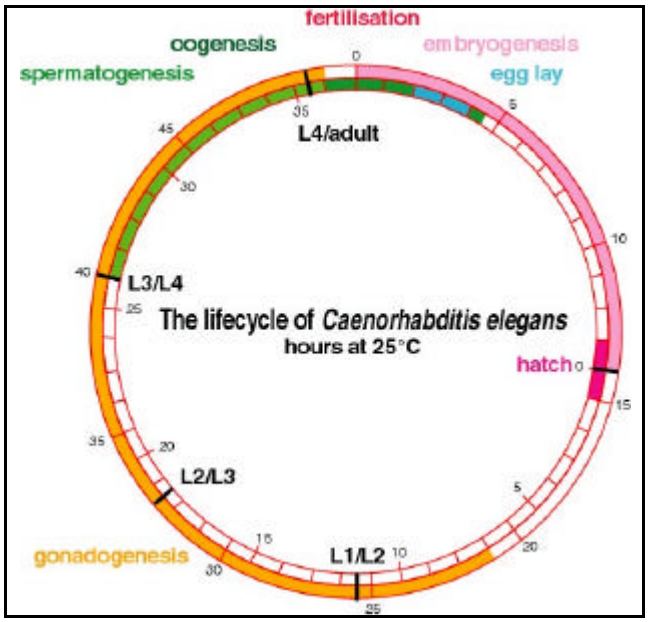


Fig. 3: The life cycle of *C. elegans*.
http://nema.cap.ed.ac.uk/Caenorhabditis/C_elegans.html

3 Ascaris species

The best-known species of the genus *Ascaris* is the so-called "large roundworm", *Ascaris lumbricoides*, which is a parasite of man, of some apes and pigs, all over the world. Controversial debates took place concerning the taxonomic status of the human and pig *Ascaris*. Scanning electron microscopy has, however, confirmed that differences do occur in the shape of lips and denticles. Some workers refer to them as separate species, *A. lumbricoides* (man) and *A. suum* (pig), or subspecies, *A. lumbricoides lumbricoides* and *A. lumbricoides suum*. Actually, it is admitted that *Ascaris lumbricoides* infects the human and *Ascaris suum*, the pig (Smyth, 1994).

3.1 Morphology

Ascaris lumbricoides (Fig. 4a and 4b) are large stout nematodes, tapering at both ends. Sexually mature male and female *Ascaris* worms typically measure 200 and 300 mm, respectively, with a width of 2-6 mm. The males are generally thinner than the females, and can also be distinguished by the prominent curvature of the posterior end (Crompton, 1989). The morphology of *Ascaris* worms reflects the general nematode body plan described by Wharton (1986). It is noteworthy, that the female parasite produces eggs with daily average output of about 200'000; up to a total of 25 millions eggs during her lifetime. The fertilized eggs are broadly ovoid, measure 50-70 x 40-50µm and possess a thick transparent light brown shell consisting of four layers (Fig.5) (Smyth, 1994).

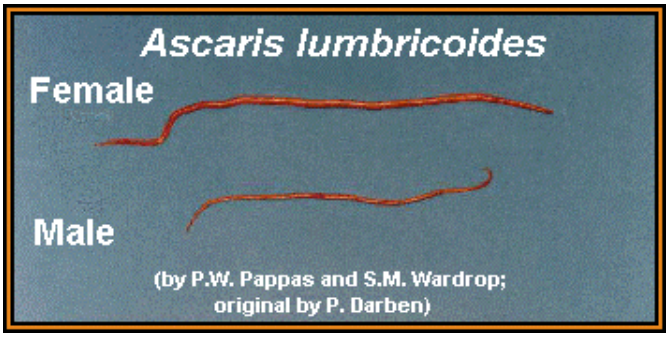


Fig. 4a: Female and male *Ascaris lumbricoides*; the female mesure approximately 30 cm in length.
http://martin.parasitology.mcgill.ca/images/ascaris.gif

Fig. 4b: A female *Ascaris lumbricoides.*
http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Ascariasis_il.htm

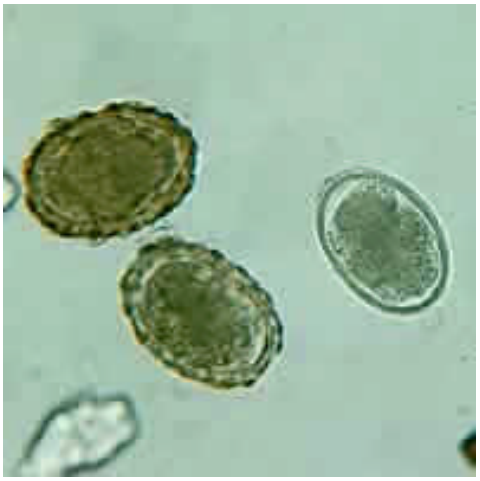


Fig. 5: Three fertilized eggs (one decorticated, on the right) of *Ascaris lumbricoides.*
http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Ascariasis_il.htm

3.2 The life cycle of Ascaris lumbricoides

About two weeks after passage in the feces, the eggs contain an infective larval or juvenile stage. Humans are infected when they ingest such eggs. The larvae hatch in the small intestine, the juvenile penetrates the small intestine and enters the circulatory system, and eventually, the lungs. In the lungs, the juvenile worm leaves the circulatory system and enters the air passages of the lungs. It usually migrates up the air passages into the pharynx where it is swallowed, and once in the small intestine, it grows into an adult worm. The life cycle is completed in about 2 months; adult worms may live 1 to 2 years (Fig.6). It is not known why *Ascaris* undergoes such a migration through the body to only end up where it started (Smyth, 1994).

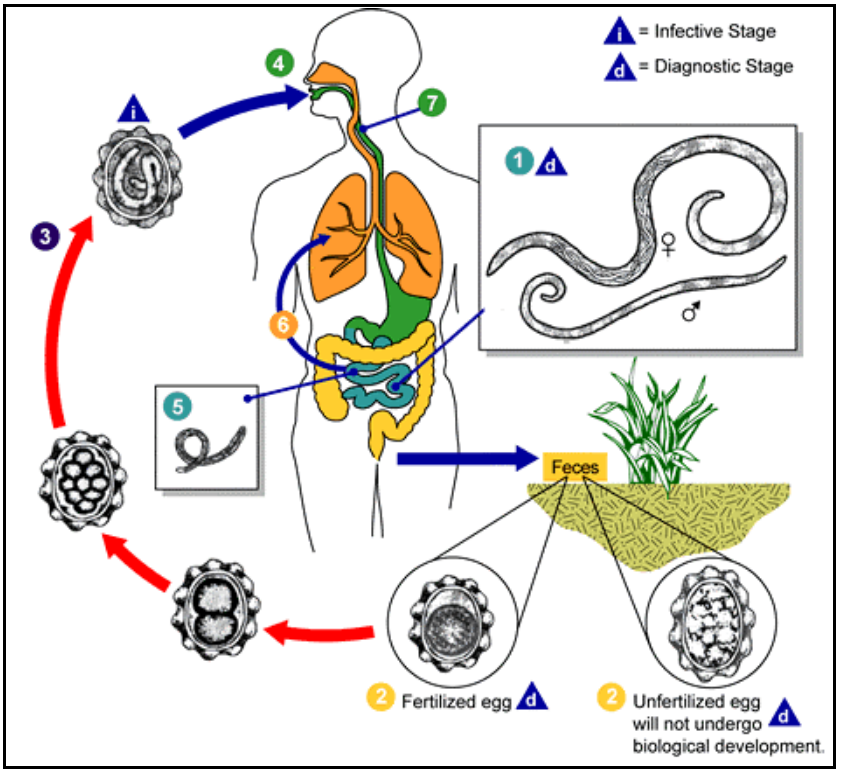


Fig. 6: Life cycle of *Ascaris lumbricoides.* Adult worms **Erreur! Argument de commutateur inconnu.**live in the lumen of the small intestine. A female may produce approximately 200,000 eggs per day, which are passed with the feces **Erreur! Argument de commutateur inconnu.**. Unfertilised eggs may be ingested but are not infective. Fertile eggs embryonate and become infective after 18 days to several weeks **Erreur! Argument de commutateur inconnu.**, depending on the environmental conditions (optimum: moist, warm, shaded soil). After infective eggs are swallowed **Erreur! Argument de commutateur inconnu.**, the larvae hatch **Erreur! Argument de commutateur inconnu.**, invade the intestinal mucosa, and are carried via the portal, then systemic circulation to the lungs **Erreur! Argument de commutateur inconnu.**. The larvae mature further in the lungs (10 to 14 days), penetrate the alveolar walls, ascend the bronchial tree to the throat, and are swallowed **Erreur! Argument de commutateur inconnu.**. Upon reaching the small intestine, they develop into adult worms **Erreur! Argument de commutateur inconnu.**. Between 2 and 3 months are required from ingestion of the infective eggs to oviposition by the adult female. Adult worms can live 1 to 2 years. http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Filariasis_il.htm

Ascaris suum life cycle is identical to that of *A. lumbricoides*. If a human ingests eggs of *A. suum* the larvae will migrate to the lungs where they are not able to survive. This can cause a particularly serious form of "*ascaris* pneumonia". Adult worms of this species do not develop in the human intestine (Smyth, 1994).

3.3 Ascariasis

Ascaris is a cosmopolitan parasite. *Ascaris* infections in humans are called Ascariasis, which is common in both tropical and temperate climate, where there is adequate moisture and low standards of hygiene and sanitation (Crompton, 1989). Ascariasis is the most prevalent intestinal helminths infection in the world; current estimates suggest that more than 1 billion persons are infected, resulting in about 20'000 deaths, each year (WHO, 2002). A clear health hazard is untreated sewage being discharged into rivers, lakes and the sea. The eggs, resistant to disinfectants, pollution and lack of oxygen, are able to continue development in sea water and remain viable for 40 days at –23°C, although they will not embryonate below 18°C. The soil type is important in transmission; clay soils favour survival of eggs, since they better retain water. Where there is a marked rise in standards of sanitation, *Ascaris* infection falls quickly. At the personal level, thorough washing and preferably cooking of vegetables and supervision of children's play areas is important. In addition, properly designed modern sewage treatement plans, such as composting and septic tanks will remove or destroy the majority of *Ascaris* eggs (Kilma, 1989).

Although the great majority of infections are symptomless, the presence of even a few worms can be potentially dangerous. The first clinical sign that may arise is a pneumonitis accompanied by cough and sometimes fever at 5-6 days after infection. There is also a moderate eosinophilia. The severity of symptoms depends both on the number of eggs ingested and on the previous infection history; hypersensitivity to infection may cause strong asthmatic symptom. The migration of the larvae through the lungs causes the blood vessels of the lungs to hemorrhage, followed by an inflammatory response accompanied by edema. The resulting accumulation of fluids in the lungs results in "*Ascaris* pneumonia" and this can be fatal. The second phase of pathogenesis results from the presence of adult worms in the intestinal lumen. The main manifestations of adult ascariasis are gastrointestinal discomfort, nausea, colic and restless sleeping. A serious complication of *Ascaris* infection is intestinal obstruction accompanied by abdominal pain, loss of appetite, and nausea. Most rare complications, such as inflammation of the bile and pancreatic ducts result from the wandering of adult parasite to these locations (Manson-Bahr and Bell, 1987; Pawlowski and Davis, 1985).

A diagnosis of Ascaris infections can be made by detection of eggs in the feces of the patient, or clinically, by the detection of an eosinophilia accompaniying the pneumonitis caused by the migrating larvae. These tests can be complemented by rather unestablished but potentially valuable diagnostic methods, such as radiography and serological diagnosis, although the latter is greatly impeded by cross reactivity with other helminthic determinants (Manson-Bahr and Bell, 1987).

Treatment is effective only against the adult worms. Although the vast majority of Ascaris infections cause few symptoms, it is however wise to treat any established infection. Most efficacious drugs are available. (Davis, 1985; Manson-Bahr and Bell, 1987). If other intestinal helminthes are present, *Ascaris* must be treated first to prevent aberrant migration of adult worms. Alleviation of obstructive complications may require surgical or endoscopic intervention

4 Filarial nematodes

The filariidae are parasitic nematodes with highly evolved cycle. All species employ arthropods as intermediate hosts. They are parasitic in all classes of vertebrates except fish. Generally speaking, filariidae are slender worms with reduced lips and oral capsule. A large number of the worms are parasites of wild animals especially of birds, several others being important disease inducers in human as well as in domestic animals (Manson-Bahr and Bell, 1987)
Filariasis is a broad term for a wide variety of symptoms that are described in the human disease resulting from infection with one of nine distinct species of nematode parasites of the class Secernentea, order Spirurida, family Filariidae. These parasites can conveniently be classified into groups according to the normal habitat of the adult worms:

1. Lymphatic filariasis: *Wuchereria bancrofti*
Brugia malayi
Brugia timori

2. Subcutaneous filariasis: *Onchocerca volvulus*
Loa loa
Mansonella streptocerca
Dracunculus medinensis

3. Serous cavity filariasis: *Mansonella perstans*
Mansonella ozzardi

4. Other (unclassified) species

Although the parasites within each group might exhibit similarities in their nature; there is a great difference between the groups. Within the topic of filarial disease, the present thesis deals with nematodes of the genus *Brugia*.

4.1 Four different lymphatic filarial worms

Adult *W. bancrofti* are thread-like white worms. The male (40 mm x 0,1 mm) is coiled, with a corkscrew-like tail and two spicules. The female (65 mm x 0,3 mm) has a tapering anterior end with a rounded swelling. Microfilariae mesure 250-300 µm x 10 µm, they are sheathed and have nocturnal periodicity (Manson-Bahr and Bell, 1987) (Fig.7).
This species causes bancroftian filariasis, resulting in elephantiasis in man, a disease inducing swellings often in the legs or in the genital system. The adult worm occurs in tightly coiled nodular masses in the major lymphatic ducts. The distribution of the worms is limited to the tropical and subtropical countries, chiefly in Asia, Africa, America and in the corresponding Pacific regions, with a long and humid intermediate–host season. It also occurs in Australia and the Mediterranean area between 40°N and 30°S (Smyth, 1994).
The most important vectors of *W. bancrofti* are members of the *Culex pipiens* “complex” in urban areas and species of *Anopheles, Aedes* and *Mansonia* (Smyth, 1994) (Fig.7d).

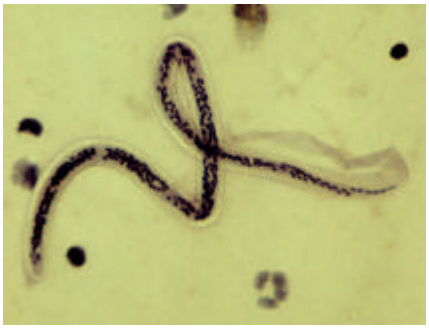


Fig. 7a: Microfilaria of *Wuchereria bancrofti*, from a patient seen in Haiti.
http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Filariasis_il.htm

Fig.7b; Female *Wuchereria bancrofti*.
http://circuit.neb.com/fgn/pnb/wuchban.html#path

Fig. 7c; Male *Wuchereria bancrofti*.
http://circuit.neb.com/fgn/pnb/wuchban.html#path

Fig. 7d: *Anopheles* sp.
http://www.biosci.ohio-state.edu/~parasite/anopheles.html

Adult worms of *Brugia malayi* parasites are white, thread-like worms. Female and males measure 55 x 0,16 mm and 23 x 0,09 mm respectively. Of particular interest is the fact that the females are viviparous and that the first stage larvae, the microfilariae, are surrounded by a sheath. The microfilariae measure 250 x 6 µm and have nocturnal periodicity. (Manson-Bahr and Bell, 1987) (Fig.8).
That species also parasites the lymph nodes and lymphatic system; the adults cause Malayan filariasis and besides man also infect monkeys, various wildcat species. Malayan filariasis is limited to South and Southeast Asia. The major vectors belong to the genus *Mansonia* and a few species of *Anopheles*. (Smyth, 1994).

Brugia timori is a species in Indonesia sharing many features with *B. malayi* but with no known animal reservoirs.

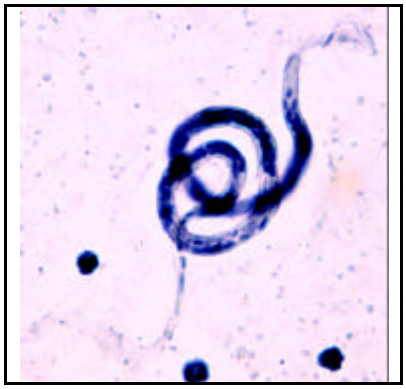


Fig. 8: Microfilaria of *Brugia malayi*.
http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Filariasis_il.htm

Brugia pahangi is a natural parasite of cats and dogs in Asia. It has been accidentally transmitted to man and might be in Borneo a natural infection in man (Palmieri *et al.*, 1985). Because it develops readily in jirds, golden hamsters and mice, it forms a valuable laboratory tool for research in filariasis.
Moreover, *Brugia pahangi* infection patterns in the cat closely parallel those of *Brugia* and *Wuchereria* in human (Smyth, 1994).

4.2 The life cycle of lymphatic filarial nematodes

Infective larvae are transmitted by infected biting arthropods during a blood meal. After entering the skin of man, the larvae migrate to the lymph glands and vessels were they moult twice, from L3 to L4, and from L4 to the adult stage. The adults dwell in various human tissues, where they can live for several years. The agents of lymphatic filariasis reside in lymphatic vessels and lymph nodes. The female worms produce microfilariae, which circulate in the blood. When the microfilariae are ingested with a blood meal by suitable mosquito species, they loose their sheath within 5-30 minutes in the stomach of the insect. They then penetrate the midgut wall and migrate to the thoracic muscles in 1-24 hours. Two days later they have moulted to the sausage-shaped second stage larvae L2. During the second week they again molt to the third stage larvae L3, which is the infective form for humans. It migrates to the head of the mosquito and enters the labium. While the mosquito feeds on the host, the larvae emerge through the tips of the labella and enter the skin of the host through the puncture wound. (Fig.9) (Manson-Bahr and Bell, 1987).

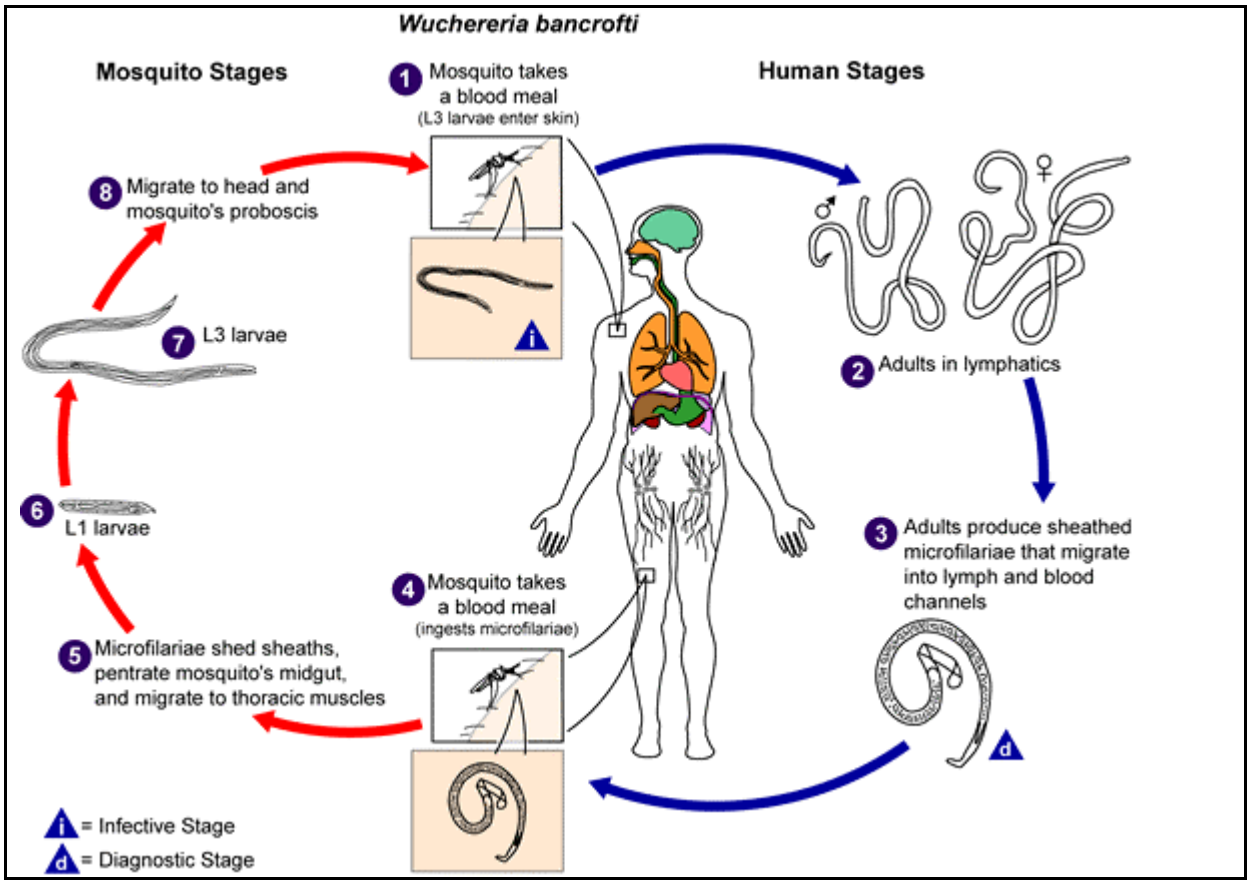


Fig. 9: Different species of the following genera of mosquitoes are vectors of *W. bancrofti* filariasis depending on geographical distribution. Among them are: *Culex* (*C. annulirostris*, *C. bitaeniorhynchus*, *C. quinquefasciatus*, and *C. pipiens*); *Anopheles* (*A. arabinensis*, *A. bancroftii*, *A. farauti*, *A. funestus*, *A. gambiae*, *A. koliensis*, *A. melas*, *A. merus*, *A. punctulatus* and *A. wellcomei*); *Aedes* (*A. aegypti*, *A. aquasalis*, *A. bellator*, *A. cooki*, *A. darlingi*, *A. kochi*, *A. polynesiensis*, *A. pseudoscutellaris*, *A. rotumae*, *A. scapularis*, and *A. vigilax*); *Mansonia* (*M. pseudotitillans*, *M. uniformis*); *Coquillettidia* (*C. juxtamansonia*). During a blood meal, an infected mosquito introduces third-stage filarial larvae onto the skin of the human host, where they penetrate into the bite wound **Erreur! Argument de commutateur inconnu.**. They develop in adults that commonly reside in the lymphatics **Erreur! Argument de commutateur inconnu.**. The female worms measure 60 to 100 mm in length and 0,24 to 0,30 mm in diameter, while the males measure about 40 mm by 100 mm. Adults produce microfilariae measuring 244 to 296 µm by 7,5 to 10 µm, which are sheathed and have nocturnal periodicity, except the South Pacific microfilariae which have no marked periodicity. The microfilariae migrate into lymph and blood channels moving actively through lymph and blood **Erreur! Argument de commutateur inconnu.**. A mosquito ingests the microfilariae during a blood meal **Erreur! Argument de commutateur inconnu.**. After ingestion, the microfilariae lose their sheaths and some of them work their way through the wall of the proventriculus and cardiac portion of the mosquito's midgut and reach the thoracic muscles **Erreur! Argument de commutateur inconnu.**. There the microfilariae develop into first-stage larvae **Erreur! Argument de commutateur inconnu.**and subsequently into third-stage infective larvae **Erreur! Argument de commutateur inconnu.**. The third-stage infective larvae migrate through the hemocoel to the mosquito's prosbocis **Erreur! Argument de commutateur inconnu.**and can infect another human when the mosquito takes a blood meal **Erreur! Argument de commutateur inconnu.**.
http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Filariasis_il.htm

4.3 Filariasis

Filariasis is a broad term for a wide variety of symptoms that are described in the human disease resulting from infection with one of nine distinct species of nematode parasites of the family *Filariidae* (see page 9) (Manson-Bahr and Bell, 1987).
Together with *W. bancrofti*, *Brugia* parasites are the causative agents of lymphatic filariasis, also known as elephantiasis, which is a highly prevalent tropical disease. It has been estimated that 1.1 billion people, in at least 80 countries, are at risk. Some 120 million people are estimated being infected. One third of those infected live in India, one third in Africa, and most of the remainder in South Asia, the Pacific and the Americas. The current global distribution of lymphatic filariasis is shown in Fig. 10.

Endemic

Non-endemic

Uncertain

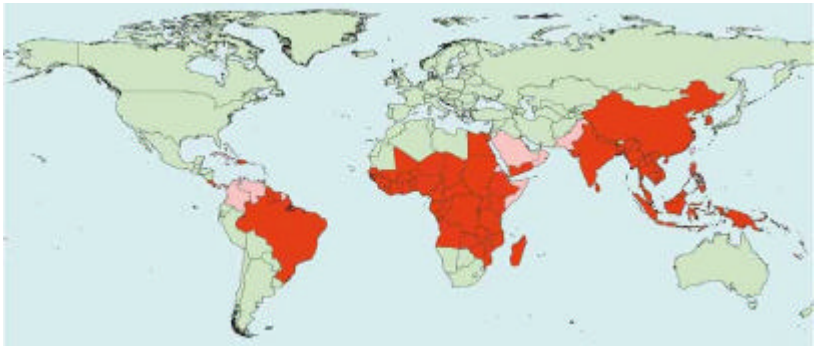


Fig. 10: Lymphatic filariasis-endemic countries.
http://www.who.int/ctd/filariasis/library/countries2.html

Globally, the infection has been recognized as the second leading cause of permanent and long-term disability, with the deforming, mutilating disease of the limbs and genitals resulting not only in physical crippling but also in serious psycho-social crippling (Fig. 11) (WHO, 2002).

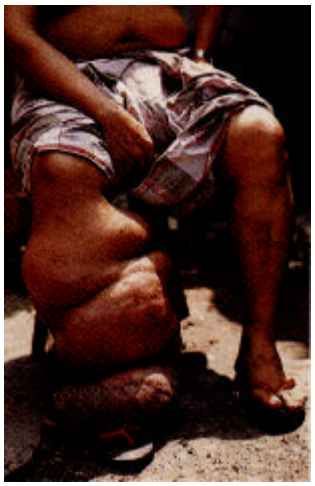


Fig. 11: Lymphatic filariasis causing Elephantiasis.
http://circuit.neb.com/fgn/pnb/filpath.html

Many cases are symptomless for years or throughout life and the only evidence of infection is the presence of microfilariae in the blood. However, from the third month after entry of the larvae there may be recurring attacks of acute lymphangitis (inflammation of the lymph ducts), with inflamed tender lymph nodes, headache, nausea and sometimes urticaria (filarial fever). The most serious clinical manifestations are due to disturbances of the lymphatic system, that may result in lymphadenitis (inflammation of the lymph nodes), lymphoedema, lymphocoeles, lymphuria chyluria (lymph containing fat in the urine) and, in males, genital lesions including hydrocoele and lymph scrotum. In a matter of years, these lymphatic lesions can lead to the grotesque disfiguring picture of elephantiasis of the limbs and the scrotum. Histological studies on autopsy and biopsy material suggest that the damage to the lymphatic vessels in elephantiasis is due to the extensive immune response to the presence of adult worms. Furthermore, successive bouts of lymphangitis lead to fibrosis of the lymphatic vessels and to the formation of lymphatic hypertension, and hence to a disturbed function (Manson-Bahr and Bell, 1987).

A further medical implication resulting from microfilariae trapped in the lungs of the host, is tropical pulmonary eosinophilia (TPE), a typical hypersensitivity reaction, that consists of recurrent episodes of asthma-like attacks that often occur at night, filarial-specific immunoglobulin E (IgE) and a marked increase in the number of eosinophilia granulocytes in blood and tissues (Manson-Bahr and Bell, 1987; Piessens *et al*,. 1990).

The classical diagnosis of lymphatic filariasis is based on direct parasitological methods, i.e. the microscopic demonstration of microfilariae in blood smears. However, microfilaraemia is not a good measure of diagnosis of filarial disease since microfilarial density does not correlate with disease severity and microfilariae may be present only in the early stages. Microfilariae may be detected in the blood by direct examination, by counting chamber, and by membrane filter techniques (Manson-Bahr and Bell, 1987). These techniques can be supplemented by a number of immunodiagnostic methods (Hamilton, 1985). Nevertheless, circulating parasite antigens can be used in serological detection of filarial infection but this will not allow establishing a sure diagnostic (Weil, 1990). The complexity of the immune response of the host against filariidae and the problems of cross-reactivity between all nematodes make not possible the interpretation of a serological diagnostic. Clearly, there is a need to develop realiable and easy-to-handle serological diagnostic field-tests for the appropriate specific detection of lymphatic filariasis.

The treatement of filariasis mainly consists of chemotherapy directed against the microfilariae. These are few existing, specific antihelminthic drugs with partially considerable side effects. The drug most used is Diethylcarbamizine (DEC), a potent microfilaricide that in addition, features some adulticidal activity. DEC interferes with the carbohydrate and folate metabolisme of filarial parasites (Subrahmanyam, 1987). Ivermectin is a further potent general microfilaricide, currently used in the mass-treatment of over two million resident of the regions covered by the Onchocerciasis Control Programme (OCP) in West Africa (Molyneux, 1995). This microfilaricide has been proposed to exert its antifilarial effect by acting on the neuromuscular system of the parasite (Campbell, 1985). The search for a drug, acting against adult filarial parasites that features limited side effects and can be used for mass treatment without medical supervision has so far been elusive. Nevertheless, amocarzine and UMF 078 are potential microfilaricidal drugs that are currently being investigated in clinical trials (Molyneux, 1995).

5 The cuticle as the nematode surface

The nematode cuticle is multifunctional. Most significant among its function is certainly the role as an exoskeleton, which defines the shape of the animal and provides a certain degree of rigidity allowing a directed movement. It also forms a barrier between the animal and its environment, providing resistance to desiccation, invasion by bacteria and fungi in free-living species and resistance to the immune system in many parasitic species.
The cuticle is synthesized and secreted by an underlying layer of hypodermal tissue that becomes activated for the synthesis of cuticle components at each postembryonic molt. During the development, there is an oscillating pattern of high rates of cuticle synthesis during molting and reduced rates in intermolt periods (Kingston, I.B., 1991). The entire cuticle is shed four times during the animal’s development and a new cuticle is formed "*de novo*" at each molt (Singh and Sulston, 1978; Cox *et al*., 1981 a).
The nematode's cuticle is an acellular matrix comprising primarily collagen, organized into different zones of distinct nature. There is no general agreement on the terminology used for describing the layers of the cuticle, but without any doubt, it consists of three layers covered by a triple-layered structure. Based on his ultrastructural and cytochemical studies, Bird (1980) has concluded, that the outer covering is a 'modified cell membrane' and referred to it as the *epicuticle*. This concept of the outside layer being a cell membrane has been disputed by Wright (1987), who points out that the surface structure is likely to vary considerably in different species as a result of different functional and anatomical requirements. Nevertheless, the term *epicuticle* now appears to be accepted for the outside layer.
Regarding the remaining layers of the cuticle, there now seems to be an agreement, that a generalized cuticle would consist of an epicuticle, a cortical zone, a medium zone and a basal zone, which overlies the epidermis (= hypodermis). The following brief description is based on the results of Bird (1984) (Fig. 12) (Smyth, 1994).

Glycocalyx — Structure composed of glycoproteins.

Epicuticle: — This structure shows great morphological variability, with a range of thickness of 6-40 nm. It consists of proteins and some lipids.

Cortical zone: These layers are referred to as 'zones': Although they contain several layers, the distinction between them is not always clear. The cortical zone is generally amorphous and its composition varies greatly between species.

Median zone: That zone is not well defined in some species and is absent in others. It often contains some fluids, in which there are fibers, struts or globular material.

Basal zone: That zone contains spiral fibers, laminae or striations; a large variety of words have been used for these structures. The fibers consist of a collagen-like protein.

Epidermis: Classically, the cell layer beneath the cuticle has been referred to as the *hypodermis*, but the term *epidermis* now appears to be preferred (Wright, 1987).

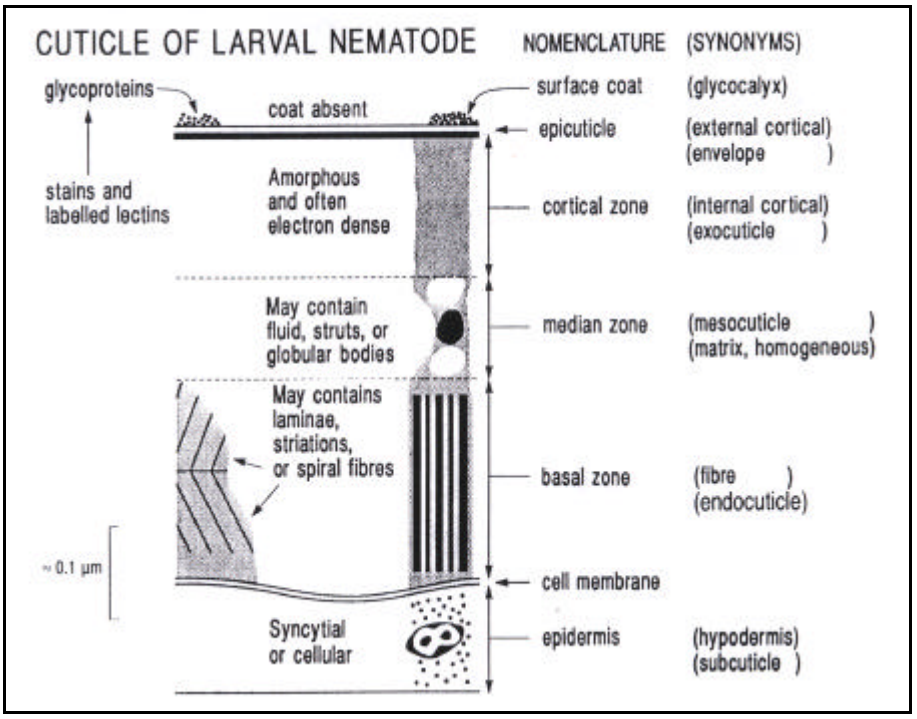


Fig. 12: Generalized diagram of the zones or layers of the cuticle of a typical larval nematode; the cuticle is ca. 0,5µm thick. Slightly different terminology has been used by different workers (After Bird and Bird, 1991) (Smyth, 1994).

Although considerable differences are found in the architecture of the cuticle between different nematode species and often between different developmental stages of the same species, all nematode cuticles appear to be similar in their basic biochemical composition. The typical nematode cuticle consists largely of proteins with small quantities of lipids and carbohydrates usually being also present (Bird, 1971).
The external epicuticle of parasitic nematodes has been reported to contain lipid components. These are organized in an osmiophilic structure.

The outer layers of the cuticle contain proteins chemically different from the collagens. These proteins constitute the remaining residual pellet observed after extensive extraction of the cuticle with detergents and reducing agents and treatment with bacterial collagenase. Fujimoto and Kanaya have termed this structure in the intestinal parasite *Ascaris lumbricoides* "cuticlin", and have shown, that, by analysis of its amino acid composition and its X-ray diffraction pattern, cuticlin is composed of a mixture of highly cross-linked proteins that clearly differ from collagen (Fujimoto and Kanaya, 1973). Amino acid analysis indicated high contents of proline and alanine, relatively low contents of glycine and basic amino acids and no hydroxyproline. The component was not reacting with collagenase, strong acids or urea and gave an X-ray diffraction pattern different from that of collagen. In contrast to the extensive molecular information on the collagenous components of the nematode cuticle (Cox, 1992), knowledge of the molecular structure of cuticlin components is almost inexistent. This is probably due to their insolubility in presence of reducing agents and denaturing detergents.

The inner cuticle layers are composed primarily of collagen-like proteins covalently linked to each other through disulfide bridges. These proteins were studied initially in *A. lumbricoides* where they were solubilized by neutral salt solutions (Josse and Harrington, 1964). Later studies showed that thiol-reducing agents could be used to solubilize collagens efficiently from isolated *A. lumbricoides* and *A. suum* adult cuticle (McBride and Harrington, 1967; Fujimoto, 1968; Winkfein et al., 1985; Betschart and Wyss, 1990). In fact, nearly 95% of the cuticle mass is solubilized by extraction in thiol-reducing and denaturing agents, such as 2-ME and SDS.

Even if the nematodes undergo four molts (the surface changes with the formation of a new cuticle), the biochemical composition of the cuticle was shown to be remarkably conserved (Politz and Philipp, 1992).

6 Molecular and biochemical aspects of cuticular collagens of nematodes

Two types of collagen proteins have been characterized in nematodes: those of the basement membrane collagen and those of the cuticle collagen. The general features of these collagen proteins as well as the corresponding encoding genes differ significantly. Nematode basement membrane collagens share many features in common with that of vertebrates. In contrast, cuticular collagens possess a unique structure, which might be a potential molecular target for immunological tools in the context of the development of immunity to nematode infections (Pritchard *et al.*, 1988).

Early studies on nematodes have shown that the major protein components of nematode cuticles are nematode-specific collagens (Josse and Harrington, 1964). Collagens are extra-cellular structural proteins with a characteristic triple-helical rod like structure, which is formed by the association of 3 polypeptide chains. Every third amino acid in the polypeptide chain must be glycine in order for the polypeptides to pack properly and to assume a triple-helical conformation. Thus, the triple-helical portion of collagen polypeptides has a repeating sequence that is commonly represented as $(Gly\text{-}X\text{-}Y)_n$ where X and Y can be any amino acid but often are proline or hydroxyproline (Cox, 1990).

The cuticular proteins encoded by collagen genes have different primary sequences, but share a common protein domain structure. They consist of about 300 amino acids, of which approximately 150 are part of repeats $(Gly\text{-}X\text{-}Y)_n$. The proteins can be divided into 5 domains (Fig.13). The sizes of the domains are similar in different nematode proteins, but not identical. Each protein contains 2 triple-helical domains and 3 non triple-helical regions. Potential cross-linking amino acids, cysteine and tyrosine residues, are located primarily in the non triple-helical domains. Domain A varies most in length and comprises in the different proteins 80-146 amino acids. Near the beginning of domain A is a stretch of hydrophobic amino acids, which presumably functions as a signal sequence for secretion. Domain A contains 3 closely spaced cysteine residues, 1 of which always is the third amino acid preceding the first $(Gly\text{-}X\text{-}Y)_n$ triplet of domain B. Domain B is a short triple-helical domain, that usually consists of 10 $(Gly\text{-}X\text{-}Y)_n$ triplets, although some genes have 9 triplets and other genes have 11 triplets. The globular domain C varies in length from 12 to 21 amino acids and contains 2 conserved cysteine residues. Certain of the genes encode a protein with a third cysteine residue at a conserved position in this domain. The large helical domain D contains 40-42 $(Gly\text{-}X\text{-}Y)_n$ triplets. This triple-helical region is interrupted near of the middle (Cox, 1990). Some proteins contain 1 or 2 additional interruptions in this region. The interruptions of the protein sequence vary from 2 to 8 amino acids in length. By analogy with vertebrate basement membrane collagens, the interruptions may serve to make more flexible the triple-helices formed by these proteins. The interruptions also may serve as sites for cross-linking. The final globular region, domain E, varies from 9 to 25 amino acids in length and contains a high proportion of potential cross-linking amino acids. This domain always contains 2 closely spaced cysteine residues, one of which sometimes is the final amino acid of domain D. Domain E also contains conserved tyrosine and lysine residues, often next to conserved cysteine residues (Cox, 1990).

Biochemical studies indicate that cysteinee cross-links play an important role in stabilizing collagens within the cuticle. Cuticle collagens typically contain 3% cysteinee residues. All of the sequenced collagens have cysteinee residues in particular locations. Each protein has three cysteinee residues in Domain A, immediately preceding the first block of $(Gly\text{-}X\text{-}Y)_n$ repeats. In addition, all of the proteins have at least two cysteinee residues in Domain C. Additional cysteinee residues are present in certain of the proteins in Domain A and C and in the $(Gly\text{-}X\text{-}Y)_n$ repeats. The positions of cysteinee residues appear to be a critical feature of the collagen polypeptides (Kramer *et al.*, 1982; Shamansky *et al.*, 1989).

In addition to disulfide links, cuticle collagens are covalently joined to one another through tyrosine-based cross-links, such as dityrosine and isotrityrosine, which involve 2 and 3 tyrosine residues, respectively (Fujimoto *et al.*, 1981; Marti, 1991; Fetterer et al., 1993). Trityrosine has not been detected, while isotrityrosine has been found only in nematodes. Dityrosine and isotrityrosine cross-links presumably are formed by the action of peroxidases, but it is not yet known where cross-linking occurs. Tyrosine-based crosslinks are probably primarily responsible for the high molecular weight forms of cuticle collagens, as other types of cross-links seen in vertebrate collagens, such as those involving lysine residues, have not been detected (Cox, 1992). Lysine residues may play a role in covalent cross-linking of collagen chains. Although some lysines are conserved in *C. elegans* collagens, the positions of lysine residues are not as conserved as those of cysteinee residues (Kramer *et al.*, 1982; Shamansky *et al.*, 1989).

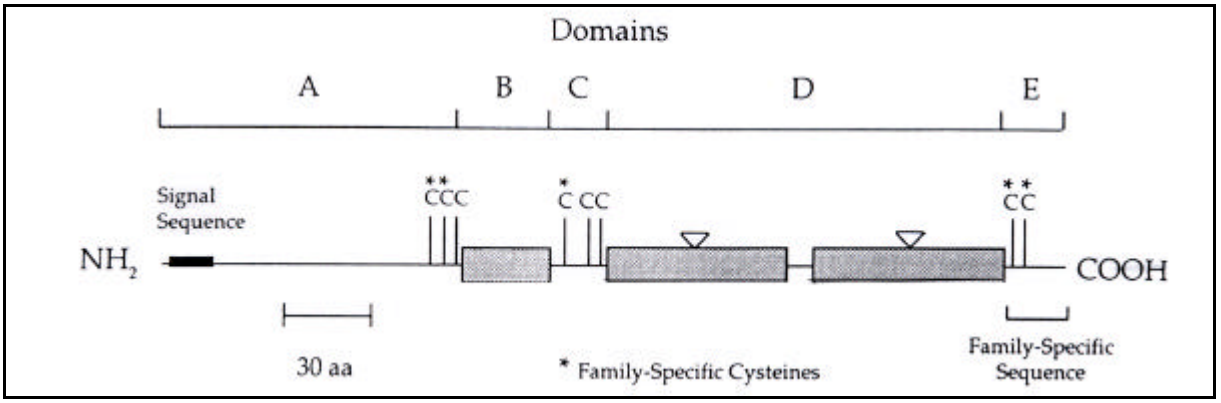


Fig. 13: Schematic representation of the general features of nematode cuticle collagens. The position of the signal sequence and $(Gly\text{-}X\text{-}Y)_n$ -triplet coding regions are indicated by solid and shaded rectangular boxes, respectively. Non-triplet-coding amino acids are indicated by horizontal lines. Triangles denote additional interruptions of the repeating $(Gly\text{-}X\text{-}Y)_n$ sequence. Cysteinee residues are indicated by vertical lines and a C (Cox, 1990).

7 Cuticular collagen gene families

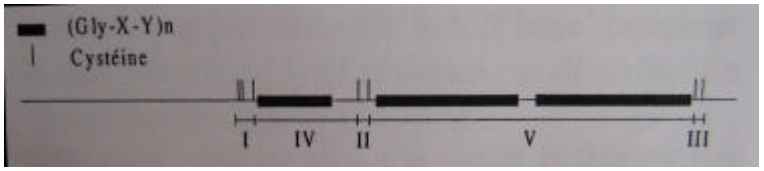
Based upon structural and sequence similarities in their proteins, nematode genes encoding cuticular collagens can be grouped into gene families (Cox, 1992). The average amino acid homology between family members is typically 60-70%. Collagens within a family are more similar to one another than to collagen of other families, regardless to which nematode species they belong. The structural features which define the families are the location of cysteine residues in domains A and E and the amino acid sequence of domain E. Collagens within a family have cysteine residues at identical positions in domains A and E and highly similar, but not necessarily identical sequences in domain E. A further family-specific feature, although not that stringent, is the conservation of tyrosine residues in domain E. The conservation of these tyrosine residues makes them prime candidates for participating in dityrosine and isotrityrosine cross-links (Cox *et al*., 1990).

The first nematode collagen genes to be isolated were cuticular collagen genes from the free-living nematode *Caenorhabditis elegans*. The complete sequence of the *C. elegans* genome indicates upwards of 150 distinct collagen genes. These genes show a common structure; they are less than 3kb and encode proteins of about 300 amino acids, their transcription gives a 1000-1500-bp mRNA. They contain only one or two short introns that distinguish them from most vertebrate introns. Nucleic acid sequence analysis of the *C. elegans* collagen genes has shown that they can be grouped into six subfamilies (Fig. 14) (Johnstone, 1993). The corresponding polypeptides differ in their overall length and in the organization of the Gly-X-Y domains, but the greatest differences are seen in the positions of the conserved cysteinee residues and in the amino acid sequences of three non-helical regions. The amino acid sequence of the carboxyl terminus appears to be particularly representative for each of the six subfamilies.

This subdivision into families is not unique to *C. elegans*. Genes that are closely related to the different proposed groups in *C.elegans* have been described for various distantly related nematode species (Johnstone, 2000). Studies were made on different nematode parasites like *A. suum* (Kingston *et al*., 1989), *H. contortus* (Shamanski *et al*., 1989), *B. malayi* (Scott *et al*., 1995), etc. These genes can also be classified into these families.

The exact way in which the triple helical structure of collagen is formed is not clear. It is now clear that the cuticular collagens have a characteristic triple-helical structure that is formed by the association of 3 polypeptide chains but no study was done on the nature of these 3 polypeptide chains. It is known that these polypeptide chains consist of about 300 amino acids but it is not known if these proteins are encoded by a unique gene. It is possible to find 3 polypeptide chains encoded by 3 different genes in the same triple helical structure. Disulphide cross-links and non reducible cross-links could occur between polypeptide chains issued from different genes. For cross-linking reasons, it seems evident that only proteins issued from the same family can bind to each other in order to pack properly and to assume the triple helical conformation. As no study was done on this subject only hypotheses can be elaborated.

A



B

Domain

Class	I	IV	II	V	III	gene
A	C1C5C (C1C6C)	40-42	C2C	133-134	C1C	*Sqt-1 (col-8)*
A-1	C2C4C	42	C2C	134	C1C	*dpy-7*
B	C0C1C	43-46	C2C	130-131	C2C	*col-6 Bpcol-1 Bmcol-2 Ascol-C*
B-1	C0C1C C0C1C	46	C1C	129-130	C2C	*col-14*
C	C2C0C	36	C7C2 (C10C)	131-132	C3C	*col-1 (col-34)*
C-1	C2C0C	36	C10C2C	131	C1C	*dpy-2*

Fig. 14: Representation of different classes of collagens. **A**. Schematic representation of cuticular collagen. **B**. Comparison of different cuticular collagen families. As described by Johnstone (1993), domains I, II and III represent the conserved clusters of cysteine residues, where cysteine residues are indicated by C and the numbers indicate the amount of amino acids between adjacent cysteine; domains IV and V contain triple helical regions plus additional non Gly-X-Y sequences.

8 Immunoparasitological consideration

A successful control of nematode infections has been so far achieved locally, but generally without permanent effects. Presently, the best form of individual prevention is avoidance of contact with the infective stage of the parasites. In the case of lymphatic filariasis this would be avoidance of mosquito bites by using repellants, mosquito nets and protective clothing, while in the case of ascariasis, an amelioration of the sanitation conditions would certainly decrease the prevalence of infection. This clearly indicates the need of further methods to control nematode infections and it seems evident that the best preventive protection would be a specific immunoprophylactic vaccine. A considerable number of scientists has been involved in immunoparasitological research giving evidence that the immune system of the host does not play a passive role during infection, but reacts with a number of immunological events (Philipp *et al.*, 1988).

By far the most often assessed function of the nematode cuticle is the excellent protection against unfavourable environmental conditions, which in the case of parasitic nematodes are the various host tissues, such as blood, lymphatics and intestines, and their associated immune responses. Therefore, the components of the cuticle are predisposed to be a potential target for a vaccine. The antigens expressed on the nematode surface are likely to play an important role in the molecular host-parasite interplay. In fact, such antigens have been shown to elicit immunological responses (Maizels and Selkirk, 1988; Betschart 1990). Already in their review of 1980, Ogilvie and coworkers have discussed the role of the complement, antibodies and cell adherence as important immunological devices to control nematode infections

(Ogilvie *et al.*, 1980). Therefore the question of the exact nature of this surface, addressed by many research workers (Betschart, 1990; Maizel *et al.*, 1993), is still of interest. A better understanding of the molecular nature of this surface is clearly required: The successful establishment of the parasite within its host clearly depends on the composition of the cuticle and on the interactions with the host immune system (Betschart, 1990; Bird and Bird, 1991). With the exception of the cuticular collagens of the non-parasitic nematode *Caenorhabditis elegans* (Cox, 1992), knowledge on the structural proteins of the cuticle of parasitic nematodes is almost inexistent. In this context, the elucidation of the molecular structure of the cuticle will increase our understanding of how surface antigens interact with the complex host environment.

As the major protein components of nematode cuticles are collagens, a study on these proteins is of great interest. In infections with filarial nematodes, antibodies to collagenous proteins of the cuticle naturally occur (Selkirk *et al.*, 1989; Selkirk and Blaxter, 1990); while the latter could be due to the exposure of these molecules to the immune system following both decay and molting events of nematode parasites, it clearly indicates the immunogenic potential of these molecules and their involvement in the immunological host-parasite interplay. This is confirmed by the finding that collagen molecules of filarial parasites are early-recognized parasite components in infections of laboratory animals (Betschart, unpublished data). This indicates the need to investigate whether variable (poorly conserved) collagen domains could be at least of potential use as (recombinant) antigens in differential diagnosis.

9 The aim of the present thesis

As outlined by Ogilvie and coworkers, the successful establishment of a parasite clearly depends on the nature and outcome of the initial and ongoing molecular interactions between the host and the parasite (Ogilvie *et al.*, 1980). In parasitic nematodes, it is the cuticle that stands in direct contact with the host environment and it is agreed that a better understanding of the host-parasite interactions that take place on the cuticle will lead to an increased knowledge on the importance of these interactions with respect to the course of disease (Maizels and Selkirk, 1988).

The study during this thesis is concentrated on cuticular collagens. It was the overall aim of the present thesis to contribute to the molecular identification of cuticular collagen components of nematodes of medical importance. In particular the specific goals of the present study were as follows:

- Comparative study of the composition of three different nematode cuticular collagens (*A. suum, C. elegans* and *B. pahangi*).
- Study of the immunogenicity and antigenicity of the cuticular collagens in order to clarify the specificity.
- Molecular study of the collagen genes.
- Production of recombinant collagens in order to study the characteristics of the immunogenicity of these collagens.
- Production of antibodies raised against the recombinant collagens in order to study the specificity.

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CHAPTER 2: COMPARISON OF CUTICULAR COLLAGEN PROTEINS OF DIFFERENT NEMATODES AND ANALYSIS OF THEIR IMMUNOGENICITY

1 Introduction

The initial biochemical studies of cuticle collagens indicated that they are a heterogenous mixture of proteins that varied in molecular weight among species and among developmental stages of the same species. McBride and Harrington (1967) also suggested that cuticle collagens formed triple-helices through a single polypeptide folding back on itself rather than through the association of 3 separate polypeptide chains. The cloning and sequencing of cuticle collagen genes revealed a quite different picture. Since the initial studies of *Ascaris* cuticle collagens, biochemical studies of cuticle collagens have been reported for several other nematodes, such as *Panagrellus silusiae* (Leushner *et al*., 1979), *C.elegans* (Cox *et al.,* 1981a), *Meloidogyne incognita* (Reddigari *et al*., 1986), *Haemonchus contortus* (Fetterer, 1989), *O. volvulus*, *Dirofilaria immitis* (Petralanda *et al*., 1991), *B. malayi* and *B. pahangi* (Selkirk *et al.,* 1989). In each nematode, cuticle collagen comprise a mixture of proteins with molecular weights ranging from 30 kDa to greater than 200 kDa, when analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The exact molecular weight of the collagen species differ in different nematodes, but generally there are minor amounts of approximately 30 kDa species, more of 60-80 kDa species, large amounts of 90-120 kDa species, and again minor amounts of higher molecular weight species (Cox, 1992). From molecular studies, it is known that cuticle collagen genes encode approximately 30 kDa proteins, which indicates that most, if not all of the higher molecular weight collagen species observed by SDS-PAGE are cross-linked aggregates of 30 kDa proteins. The 60-80 kDa species and the 90-120 kDa species represent 2 and 3 cross-linked collagen chains, respectively (Betschart and Wyss, 1990). The true picture is likely to be more complicated, because the exact molecular weights of the collagen species are expected to vary depending upon which cuticle collagen genes are expressed, the relative amount of cross-linking between proteins, and the location of the cross-links in the polypeptide chains. Moreover it seems that additional cross-links allow the association of polypeptide chains in a head-to-tail or a head-to-head orientation. These long fibers interact with each other to form the multilayer cuticle (Cox, 1992). All these parameters influence the migration properties of collagens in polyacrylamide gels.

In this chapter, studies are focused on the comparison of the protein profiles of three different nematode cuticular collagens: *A. suum, C. elegans* and *B. pahangi.* As no comparison of these three different protein profiles was published till today, this analysis is done in order to verify the presence of the three forms (monomer, dimer, trimer) in these three species and also to verify the presence of cross-links between the polypeptide chains. The collagenous nature of these cuticular proteins is also demonstrated using enzymatic digestion by collagenase and pepsin.

The second part of this chapter is based on the immunogenicity and the antigenicity of the cuticular collagens in order to clarify the specificity. As the cuticle of nematodes is in direct contact with the host, an immunological study on the cuticle seems to be interesting. As mentioned in the general introduction, the major protein components of nematode cuticles are nematode specific collagen. Although the cuticular collagens are usually not directly exposed to the host immune system, some collagenic fragments from the old cuticle are present in blood during each molt, therefore accessible to the cells of the immune system. For that reason the studies of the immunogenicity of cuticular collagen are undertaken. Any substance eliciting an immune response is said to be immunogenic and is called an immunogen. There is a clear operational distinction between an immunogen and an antigen. An antigen is defined

as any substance that can bind a specific antibody. All antigens therefore have the potential to elicit specific antibodies, but some need to be attached to an immunogen in order to do so. This means that although all immunogens are antigens, not all antigens do possess immunogenic properties (Janeway, Travers, Walport and Capra, 1994).

The specificity of the cuticular collagen antibodies is also of interest. Antisera contain many different antibody molecules that bind to the immunogen in slightly different ways. Some of them are cross-reactive. A cross-reaction is defined as the binding of an antibody to an antigen other than the immunogen; most of them generally cross-react with closely related molecules. Some of them indicate the presence of cross-reactivity. These cross-reacting antibodies can create problems, when the antiserum is used for the detection of a specific antigen. This is one reason to study the specificity of cuticular collagen antibodies. Moreover, it is known (Selkirk *et al.*, 1989) that antibodies against cuticular collagens have been detected in patients infected with nematode parasites. The production of antibodies raised against *A. suum* cuticular collagens, the study of their specificity and the study of cross-reaction with cuticular collagen of other nematodes will be discussed.

2 Materials and methods

2.1 Parasites

Adult *Ascaris suum* worms were received from a local slaughterhouse and stored at –20°C.
The *C. elegans* worms (strain Daf 7; kindly provided by Dr. Fritz Müller, University of Fribourg, Switzerland) were grown according to the method described by Sulston & Brenner (1974).
Brugia pahangi were kindly provided by Dr. E. Deveney (Department of Veterinary Parasitology, University of Glasgow, UK).

2.2 Protein extraction

The cuticles from adult *A.suum* were isolated by freezing the worms several times and then removing the cuticle mechanically (Fujimoto and Kanaya, 1973; Marti, 1991). The cuticle fragments were cut into pieces and incubated 2 minutes at 100°C, then treated overnight at room temperature in SDS 1% and 125*mM* Tris-HCl pH of 6,8. Cuticles were then centrifuged at 4°C (15 minutes at 10'000g). The residue was boiled during 2 minutes (in 125*mM* Tris-HCl pH 6,8; 1% SDS; 5% 2-ME) before its incubation overnight at room temperature. The supernatant containing the collagen proteins was recovered after centrifugation at 4°C (15 minutes at 10’000g) and dialyzed overnight in 5% 2-ME (Cox *et al*., 1981a; Betschart, 1990).
C. elegans were briefly washed in cold phosphate buffered saline (140 *mM* NaCl, 100*mM* phosphate buffer pH 7,4), sonicated in 125*mM* Tris HCl, pH 6,8, 1*mM* EDTA, 1*mM* PMSF, 1% SDS, 5% 2-ME and stirred for 4 hours at 4°C. The extraction of the collagen proteins was made as for *A. suum* (Cox et *al*., 1981a).
Cuticular proteins of *Brugia pahangi* were prepared by Bisoffi (1995). The protocol was the same used for *C. elegans*. However the proteins correspond to a soluble extract of nematodes and not to collagen extract only.

2.3 Sample of proteins

The quantification of the proteins was made by gravimetry. The protein samples were dialysed, freeze-dried, weighted and resuspended into the appropriate buffer (125*mM* Tris-HCl pH 6,8; 1% SDS; 5% 2-ME) at a concentration of 1mg/ml.

2.4 SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

Soluble collagens of *A. suum* (1mg/ml) were analyzed by polyacrylamide gel electophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE, MiniGel: Bio Rad Laboratories). Samples prepared under reducing condition were boiled for 3 minutes in SDS polyacrylamide

gels sample buffer (62,5 *mM* Tris-HCl, pH 6,8, 10% glycerol, 2% SDS, 5% 2-ME). Collagen proteins were loaded onto the gel. Each well contained 15µg of cuticular collagen. The stacking gel was composed of 3,5% acrylamide and the separating gel 12% acrylamide. Electrophoresis was carried out at 100-150 Volts in electrode running buffer (25 *mM* Tris; 192*mM* glycine, 0,1% SDS; pH 8,3) until the coloured dye contained in the loading buffer (Sambrook, *et al*., 1989) reached the end of the gel. One well contained 5µl of a molecular weight marker (Bio Rad, Broad range molecular marker).
After fixation of the proteins (25% Isopropanol, 10% Acetic acid), the gel was stained with Coomassie blue solution (0,1% Coomassie blue in 50% Methanol, 10% Acetic acid). Finally the detection of separated protein bands was obtained by fading the gel in a solution of 5% methanol, 7% Acetic acid (Sambrook *et al*., 1989).

2.5 Collagenase treatment

High purity collagenase from *Clostridium histolyticum* (Sigma type VII, Switzerland) was used for the digestion of the dissolved proteins. 15 µg of collagen proteins were treated with 0,6 µg of enzyme and the experiment was performed for various periods of time at 37°C in 10 *mM* Tris-HCl, pH 7,5, 10 *mM* calcium chloride (Selkirk *et al*. 1989).
The reactions were terminated by addition of a protein loading buffer (62,5 *mM* Tris-HCl, pH 6,8, 10% glycerol, 2% SDS, 5% 2-ME) followed by heating the samples for 2 minutes at 100°C. Samples were submitted to usual electrophoresis.

2.6 Pepsin treatment

0,6 µg of Pepsin from hog stomach (Fluka) was used to treat 15 µg of collagen protein. The reaction was performed for various times at 37°C in 10 *mM* Tris-HCl, pH 7,5, (Selkirk *et al*. 1989).
Digestions were terminated diluting the samples into protein loading buffer (62,5 *mM* Tris-HCl, pH 6,8, 10% glycerol, 2% SDS, 5% 2-ME) and heating the samples for 2 minutes at 100°C. Samples were submitted to usual electrophoresis.

2.7 Antisera

A polyclonal antiserum raised against cuticular collagen proteins of adult *A. suum* was produced in Balb/c mice using cuticular collagen extracts of adult *A. suum*, prepared according to Fujimoto and Kanaya (1973) and isolated from polyacrylamide gels (Marti, 1991).
A polyclonal antiserum raised against cuticular collagen proteins of adult *B. pahangi* nematodes was provided by Dr. M.E. Selkirk, Imperial College of Science, London, UK. The cuticular collagens from adult *B. pahangi* were isolated essentially as described for *C. elegans* (Cox *et al*., 1981a). The soluble material was used to immunize rabbits according to standard protocols (Maniatis *et al*., 1982). This polyclonal antiserum was named *B. pahangi* (a-205).

An affinity-purified anti-*Bpcol-1* antibody, named *Bpcol-1* (a-19-1) was kindly provided by Bisoffi (1995). The denomination 19-1 corresponds to the clone used to produce the recombinant cuticular collagen protein *Bpcol-1*. The clone (19-1) codes for cuticular collagens.
Human sera from infected patients kindly provided by Yves Moosman from the Diagnostic Parasitaire, University of Neuchâtel, Switzerland. The list of the sera used in this work is presented in Table 1.

Name	**Primary antibodies**	**Secondary antibodies** (Nordic Immunological Laboratory)
a-30colA.s	Mouse/anti *A.suum* cuticular collagen 30kDa	Goat/anti-mice (IgG, H+L) peroxidase
a-50-70colA.s	Mouse/anti *A.suum* cuticular collagen 50-70kDa	Goat/anti-mice (IgG, H+L) peroxidase
a-120colA.s	Mouse/anti *A.suum* cuticular collagen 120kDa	Goat/anti-mice (IgG, H+L) peroxidase
CD68	Human (*Ascaris lumbricoides*)	Goat/anti-human (IgG, Fc) peroxidase
Bpcol-1 (a-19-1)	Mouse/anti *Brugia pahangi* cuticular collagen specific gene *Bpcol-1*	Goat/anti-mice (IgG, H+L) peroxidase
B. pahangi (a-205)	Rabbit/anti *Brugia pahangi* cuticular collagen	Goat/anti-rabbit (IgG, H+L) peroxidase

Table 1: List of primary and secondary antibodies that were used during the research.

2.8 Purification of proteins by electroelution.

The Schleicher & Schuell Biotrap® system is an electroseparation method designed for the rapid isolation and purification of macromolecular samples from fractionating gels or solutions. In that system, protein solutions are filtered through a set of selective membranes under the driving force of an electric field. This process yields highly pure, concentrated isolates.
The gel containing the requested proteins is separated from the support by the aid of a scalpel and introduced into the gutter of the Schleicher & Schuell system (Bio Rad). The gutter contains two different membranes. The BT1 membrane is impermeable to proteins and the BT2 contains pores of 0,8µm. The electroelution was made according to the protocol delivered by the distributor of the system. After the migration (16 hours at 100volts) in electrode running buffer (25 *mM* Tris; 192*mM* glycine, 0,1% SDS; pH 8,3) the purified protein solutions were recovered from the accumulation compartment.
The proteins were then precipitated with acetone (5X the initial volume). After 15 min centrifugation at 4°C, the pellet of proteins was weighted and then suspended in 1X PBS buffer (140*mM* NaCl, 100*mM* phosphate buffer pH 7,4) at a final concentration of 1mg/ml and conserved at –20°C.

2.9 Immunization

The production of antibodies is made using Balb/c mice (animalhouse from the Institute of Zoology of Neuchâtel).
Before the immunization process, a blood sample of 100µl was taken from the mice as a negative control by a retro orbital puncture. Balb/c mice were immunized by a subcutaneous injection of 100µg antigen in Freund's incomplete adjuvant followed by another similar subcutaneous immunization every 2 weeks. The blood was recovered 2 months after the initial immunization by bleeding the mice. The blood was then incubated at room temperature during a few hours and then centrifuged for 10 minutes at 3000 rpm. Sera containing the antibodies were recovered and frozen at –20°C.
To produce antibodies raised against cuticular collagen of *A. suum*, the mice were immunized separately with cuticular collagen proteins of different molecular sizes. Three kinds of sera were produced: sera containing antibodies raised against cuticular collagen from 90-120 kDa, named a-120colA.s, sera containing antibodies raised against cuticular collagen from 50-70 kDa, named a-50-70colA.s, and sera containing antibodies raised against cuticular collagen from 30 kDa, named a-30colA.s.

2.10 Immunodetection of cuticular collagens

Transfer of proteins to cellulose nitrate 0,45µm membrane (Schleicher & Schuell BA85) was accomplished by applying 2mA/cm^2 of gel, for 45 minutes, in a solution of 20 *mM* Tris-HCl pH 7,5, 25 *mM* Glycine 1,3 *mM* SDS, 20% Methanol (Lämmli, U.K., 1970; Sambrook, *et al.*, 1989). After transfer (Trans-Blot Semy-Dry Transfer Cell, Bio Rad) the non-specific sites were blocked by incubating the membrane for 45 min at room temperature with 5% powdered milk (Rapilait Migros) diluted in TBS. The membrane was incubated overnight at room temperature in a solution of TBS-1% powdered milk containing the primary antibody at a final concentration of 1:100. After washing twice with TBS plus 0,05% Tween, the antigen-antibody complexes on the nitrocellulose paper were analyzed by secondary antibodies. The membrane was incubated for 2 hours at room temperature with a second antibody coupled to peroxidase (Nordic Immunological Laboratory) diluted 1:1000 in TBS-1% powdered milk. After washing, the revelation was performed with a colouring solution. Finally the membrane was washed with water and dried. All steps were performed at room temperature with moderate shaking.

TBS (Tris Buffered Saline):	20 *mM* Tris-HCl pH 7,5; 500 *mM* NaCl
TTBS (Tween TBS):	TBS; 0,05% Tween-20
Powdered milk-TBS 1 % and 5%:	TBS; 1% and 5% of powdered milk
Colouring solution:	TBS; 500µg/ml 4-chloro-1-naphtol (in Methanol); 0,015% H_2O_2

3 Results

3.1 The extraction of A. suum cuticular collagen proteins

The analysis by SDS polyacrylamide gels from samples of different extraction stages of *A. suum* cuticular collagens gives very different protein profiles. After a first treatment with SDS, the obtained profile of the supernatant gives a lot of bands from 20 kDa to more than 200 kDa. Stronger bands are visualized around 200 kDa and 116 kDa. The second sample corresponding to the supernatant after treatment with 2-ME gives a band of 30 kDa, a series of bands aroud 60-90 kDa and a series of bands around 120-200 kDa. The second treatment with 2-ME reveals three main bands of 30, 66 and 120 kDa. Other bands appear but less strongly. The band of 200 kDa appears very weakly (Fig. 1).

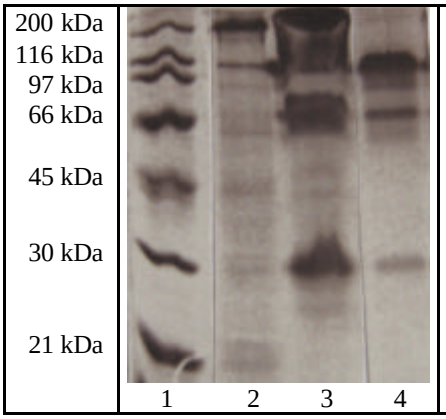

Fig.1: Polypeptide pattern of cuticular extracts of *A. suum* after separation on 12 % SDS-polyacrylamide gels. Lane 1: Marker; lane 2: Cuticle treated with SDS 10 %; lane 3 and 4: First and second treatment with 2-ME (Fujimoto and Kanaya, 1973). The values in kDa correspond to the size of the molecular marker (Bio-Rad, Broad range molecular marker).

3.2 Comparison of soluble cuticular protein profiles of A. suum, C. elegans and B. pahangi

Analysis by electrophoresis on 12 % sodium dodecyl sulfate polyacrylamide gel of the protein profiles for the parasites *A. suum*, *B. pahangi* and the free-living nematode *C. elegans* was carried out (Fig. 2). The protein extracts were prepared according to the protocols described in materials and methods (see page 28).

Among *A. suum*, the protein profile allows to distribute the proteins into 3 different groups corresponding to the collagen sizes: 30 kDa, 50-70 kDa, 90-120 kDa. A weaker band appears around 200 kDa. In comparison with the protein profile of *A. suum* different bands appear for *C. elegans* and *B. pahangi*. Among *C. elegans*, the main bands appear around 90-120 kDa. A band is also present around 200 kDa. Weaker bands are present around 50-70 kDa. No band appears at 30 kDa. Among *B. pahangi*, a lot of supplementary soluble proteins are present. A digestion with collagenase will confirm the nature of these bands. However, series of bands appear in the four groups described in the introduction. The bands around 200 kDa are more visible than those observed among *A. suum*. A supplementary band is present around 45 kDa.

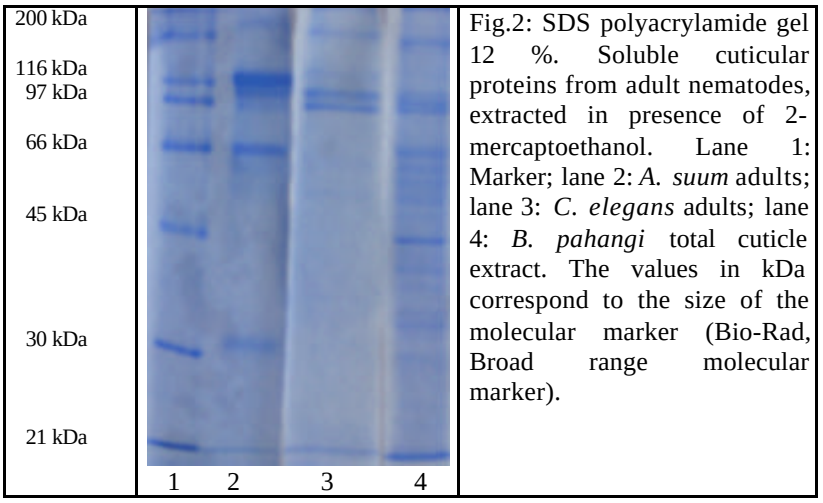

Fig.2: SDS polyacrylamide gel 12 %. Soluble cuticular proteins from adult nematodes, extracted in presence of 2-mercaptoethanol. Lane 1: Marker; lane 2: *A. suum* adults; lane 3: *C. elegans* adults; lane 4: *B. pahangi* total cuticle extract. The values in kDa correspond to the size of the molecular marker (Bio-Rad, Broad range molecular marker).

3.3 Collagenase and pepsine digestion

The analysis by SDS polyacrylamide gels of soluble cuticular proteins of *A. suum*, *B. pahangi* and *C. elegans* shows different profiles after digestion with collagenase or pepsine (Fig.3).
In *A. suum*, the bands around 90-120 kDa are no longer present after the digestion with collagenase. The bands around 50-70 kDa are still present but the intensity of the bands is weaker. Moreover a lot of supplementary bands appear from 20-50 kDa, bands that were not present before the digestion with collagenase. For the digestion with pepsine, the bands present in the four main groups discribed in the introduction are still present but the intensity of the bands is weaker. Moreover a lot of different bands appear from 30-120 kDa.
In *C. elegans*, the digestion with collagenase shows that the 200 kDa bands and the bands around 90-120 kDa are weaker than those observed before the digestion. As for *A. suum*, a lot of supplementary bands appear in the smaller molecular weights. The digestion with pepsine shows bands of weaker intensity around 120 kDa.
In *B. pahangi,* the digestion with collagenase shows that the 200 kDa bands are not present after the digestion. The band around 45 kDa is digested with collagenase. The bands around 90-120 kDa and 50-70 kDa are weaker but still present. As for *A. suum* and *C. elegans*, a lot of supplementary bands appear in the smaller molecular weight range. Except in the intensity of the bands, the digestion with pepsine shows no difference between the proteins that are not digested with those that are digested.
The sensibility of the proteins towards collagenase and pepsine digestion confirms that the proteins extracted corresponded to cuticular collagens having a polar and triple helical helix. As a negativ control, the marker was also digested with collagenase and pepsin.

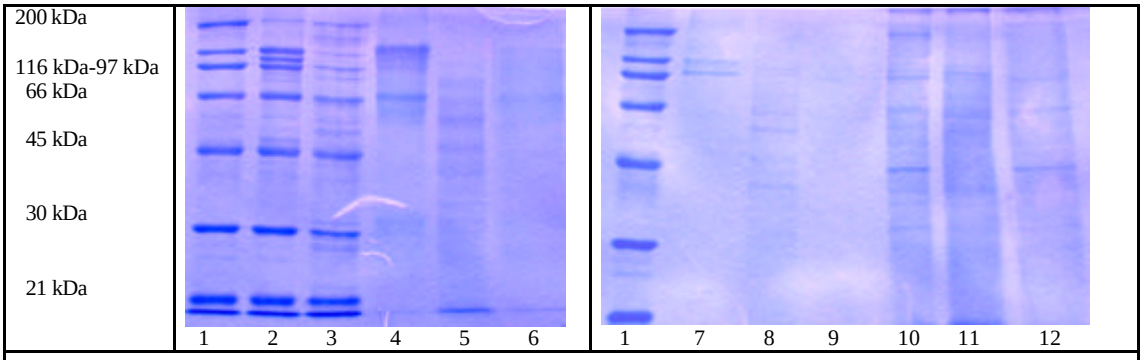

Fig. 3: Collagenase and pepsin digestion. Lane 1: Marker; lanes 2-3: Marker digested respectively with collagenase and pepsin; lanes 4-5-6: *A. suum* collagen protein profiles; lanes 7-8-9: Collagen protein profiles of *C. elegans*; lanes 10-11-12: Cuticular protein profiles of *Brugia pahangi;* lanes 5,8,11: Proteins digested with collagenase. Lanes 6,9,12: Proteins digested with pepsin. The values in kDa correspond to the size of the molecular marker (Bio-Rad, Broad range molecular marker).

3.4 Immunoblot on A. suum cuticular collagens using several produced antisera

Antiserum a-120colA.s, produced via *A. suum* cuticular collagen of 120 kDa, recognizes different *A. suum* cuticular collagen proteins of different sizes. Numerous bands were observed around 90-120 kDa and two bands around 60-66 kDa. Two bands appear around 40-45 kDa. A weaker band appears at 30 kDa. For the immunoblot made with the primary antisera a-50-70colA.s and a-30colA.s the same bands are recognized. The reactions are very weak with the antiserum a-30colA.s (Fig. 4).
In parallel, immunoblot on *A.suum* cuticular collagen digested with collagenase were made with the three different antisera (Fig. 5). The antiserum a-120colA.s still reacts with proteins with molecular weight around 60-66 kDa, 45 kDa and 30 kDa. Same results are observed with the antisera a-50-70colA.s and a-30colA.s. However the reactions are very weak with antiserum a-30colA.s.

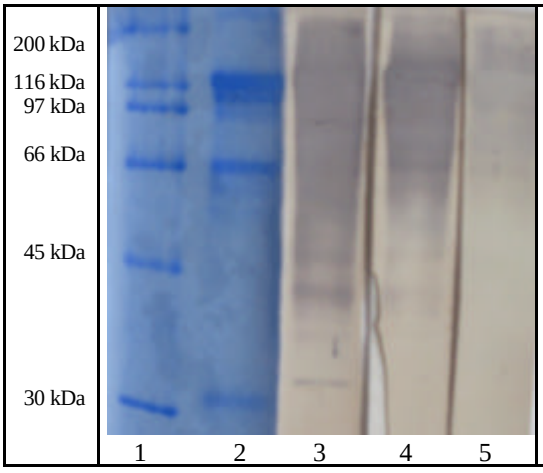

Fig. 4: Immunoblot on *A. suum* cuticular collagen. Lane 1: Marker; Cuticular collagens of *A. suum* were analyzed in SDS-PAGE (lane 2) and electroblotted onto nitrocellulose (lanes 3-4-5). Immunoblots were performed using a-120colA.s (lane 3), a-50-70colA.s (lane 4) and a-30colA.s (lane 5). The values in kDa correspond to the size of the molecular marker (Bio-Rad, Broad range molecular marker).

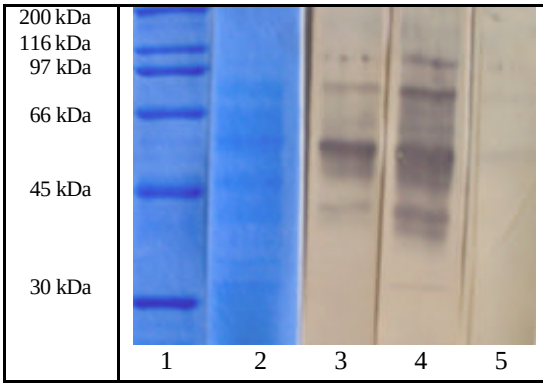

Fig. 5: Immunoblot on *A. suum* cuticular collagen digested with collagenase. Lane 1: Marker; *A. suum* cuticular collagens digested with collagenase were analyzed in SDS-PAGE (lane 2) and electroblotted onto nitrocellulose (lanes 3-4-5). Immunoblot were performed using a-120colA.s (lane 3), a-50-70colA.s (lane 4) and a-30colA.s (lane 5). The values in kDa correspond to the size of the molecular marker (Bio-Rad, Broad range molecular marker).

3.5 Crossreactions of different antisera

Immunoblot on *A. suum* cuticular collagens with a-120colAs and *B. pahangi* (a-205) show bands around 90-120 kDa, 60-66 kDa and 30 kDa. The reaction is stronger with *B. pahangi* (a-205) where additional bands appear from 40-60 kDa. The bands appear less intensively with human sera and the band around 30 kDa is not visible. No reaction is formed with *Bpcol-1* (a-19-1) (Fig. 6).

Immunoblot on *C. elegans* cuticular collagens with *B. pahangi* (a-205) show a lot of reactions around 60-200 kDa. Reaction with a-120colAs serum shows 2 bands around 60-66 kDa and 3 bands around 90-120 kDa. A band around 30 kDa is also present. Reaction with human sera reacts around 60-66 kDa and weakly around 90-120 kDa. A band is also present around 30 kDa. No reaction appears with the serum *Bpcol-1* (a-19-1) (Fig. 7).

Immunoblot on *B. pahangi* cuticular collagen show strong reaction with *B. pahangi* (a-205) around 90-120 kDa, 60-66 kDa, 45 kDa and 30 kDa. Reaction around 120 and 66 kDa appears with *Bpcol-1* (19-1) (Fig.8).

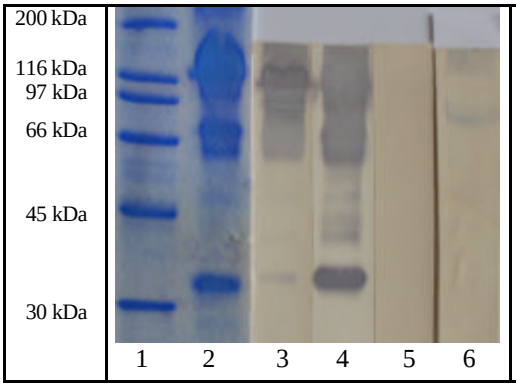

Fig. 6: Immunoblot on *A. suum* cuticular collagen. Lane 1: Marker; Cuticular collagens of *A. suum* were analyzed in SDS-PAGE (lane 2) and electroblotted onto nitrocellulose (lanes 3 to 6). Immunoblot were performed using a-120colA.s (lane 3), *B. pahangi* (a-205) (lane 4), *Bpcol-1* (a-19-1) (lane 5). Lane 6 corresponds to the immunoblot obtained with human sera CD 68. The values in kDa correspond to the size of the molecular marker (Bio-Rad, Broad range molecular marker).

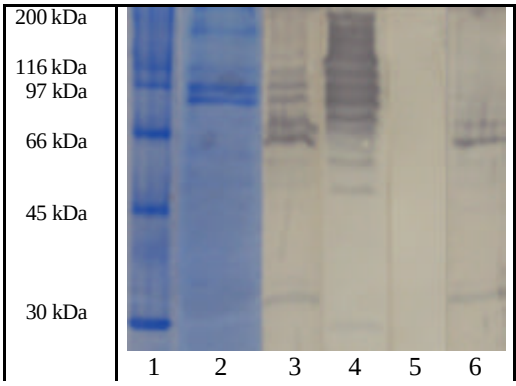

Fig. 7: Immunoblot on *C. elegans* cuticular collagen. Lane 1: Marker; Cuticular collagens of *C. elegans* were analyzed in SDS-PAGE (lane 2) and electroblotted onto nitrocellulose (lanes 3 to 6). Immunoblot were performed using a-120colA.s (lane 3), *B. pahangi (*a-205) (lane 4), *Bpcol-1* (a-19-1) (lane 5). Lane 6 corresponds to the immunoblot obtained with the human sera CD 68. The values in kDa correspond to the size of the molecular marker (Bio-Rad, Broad range molecular marker).

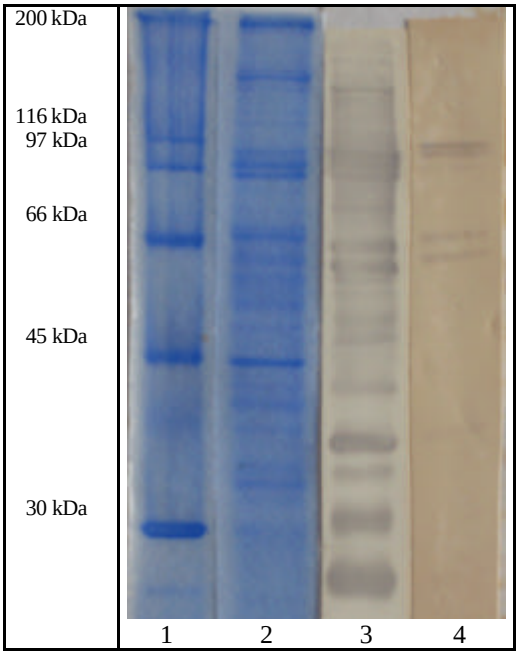

Fig. 8: Immunoblot on *B. pahangi* cuticular proteins. Lane 1: Marker; Cuticular collagens of *B. pahangi* were analyzed in SDS-PAGE (lane 2) and electroblotted onto nitrocellulose (lanes 3-4). Immunoblot were performed using *B. pahangi* (a-205) (lane 3), *Bpcol-1* (a-19-1) (lane 4) The values in kDa correspond to the size of the molecular marker (Bio-Rad, Broad range molecular marker).

4 Discussion

4.1 Comparative study of the protein profiles of different cuticular collagens

The analysis by SDS polyacrylamide gel of the extracted collagens from adult *A. suum* shows that the major structural components of the cuticular matrix consist of a set of proteins of various molecular weights ranging from 30 kDa to greater than 200 kDa. The exact molecular weights of the collagen species differ between the three different nematodes, but the predominant ones are usually between 90 and 120 kDa. These results are in accordance with those observed by Betschart and Wyss, (1990). They observed that the extracted collagen from adult *A. suum* can be separated by SDS polyacrylamide gel into four major groups of polypeptides with apparent molecular masses of 30 kDa, 50-70 kDa, 90-120 kDa and >200 kDa. They also demonstrated that a densitometric evaluation shows a proportional relationship between these four groups of 1:2:6:1 with the 90-120 kDa group as the most prominent one. They concluded, that in the cuticles of adult worms of *A. suum* the collagen molecules are present in the form of monomers, dimers and trimers. The monomers are the 30 kDa polypeptides found in the SDS polyacrylamide gel, the dimers are represented by the group of polypeptides between 50-70 kDa and the trimers by the group of 90-120 kDa polypeptides. In addition to disulfide cross-links, cuticular collagens are covalently joined to one another through tyrosine-tyrosine cross-links. These non-reducible cross-links must probably occur between polypeptides in different triple-helices in order to account for the molecular weight species >200 kDa observed by SDS polyacrylamide gel (Cox 1992). Therefore tyrosine cross-links are primarily responsible for the high molecular weight forms of cuticle collagen proteins detected by SDS polyacrylamide gel. (Cox, 1992).
It has been shown that cuticular collagen genes encode approximately 30 kDa proteins, confirming that most, if not all, of the higher molecular weight collagen species observed by SDS polyacrylamide gel are cross-linked aggregates of 30 kDa proteins (Betschart and Wyss, 1990).
The comparison of the protein profiles of *C. elegans* and *B. pahangi* with those of *A. suum* reveals similarities:

1. The major structural components of the cuticular matrix consist of a set of proteins of various molecular weights ranging approximately from 30 kDa to greater than 200 kDa.
2. The four major groups of polypeptides described for *A. suum* are also present for *C. elegans* and *B. pahangi.* The SDS polyacrylamide gel analysis of *C. elegans* does not allow confirming the presence of collagen around 30 kDa. However immunoblots show the presence of a band around 30 kDa. A collagenase digestion should be done to confirm the nature of this band.
3. The principle of monomer, dimer and trimer is also present in *C. elegans* and *B. pahangi* and the principle of disulfide cross-links and non reducible cross-links are also valid.

Also Cox *et al.*, (1981) were not able to demonstrate proteins around 30 kDa in *C. elegans.* They have demonstrated that the cuticular soluble proteins of *C. elegans* adults are always above 50 kDa. Smaller proteins were however isolated among larvae. The nature of these proteins is still unknown, but it seems that these proteins are resistant to collagenase digestion (Cox *et al,*. 1981).
As the molecular studies made on *C. elegans* cuticular collagen genes demonstrated that their genes encode also approximately 30 kDa proteins it can be concluded that all proteins are rapidly cross-linked into aggregates of > than 30 kDa proteins.
An important difference is also present in the densitometric evaluation showing a proportional relationship between the four groups of polypeptides. The values observed by Betschart and Wyss (1990) for *A. suum* do not correspond with the intensity of the protein profile of *C. elegans* and *B. pahangi*. No densitometric evaluation was done during this work but a first observation allows concluding that the proportion 1:2:6:1 is not correct for *C. elegans* and *B. pahangi*.
Another difference is the fact that bands around 40-50 kDa are present in *C. elegans* and *B. pahangi*. The collagen nature of these soluble cuticular proteins was confirmed by the use of enzymes like collagenase or pepsine. As mentioned by Cox (1990), all soluble cuticle proteins of the adult nematodes are degraded by bacterial collagenases, thus indicating their origin. A digestion with collagenase destroys all the helical zones of the collagen, therefore providing a different protein profile on SDS polyacrylamide gel.
As the pepsin destroys all globular regions of cuticular collagen proteins, a change in the protein profiles after treatment with pepsine indicates the cuticular nature of these collagens. Cox observed that treatment of the proteins with pepsin reduces the molecular weights of the 2 major collagen species from 100 and 90 kDa to 77 kDa, which corresponds to a decrease of 14 to 23% of the molecular weight (Cox 1992).
Results of these digestions on collagen proteins allow confirming the cuticular collagen nature of these proteins.

4.2 Immunogenicity of A. suum cuticular collagens

The production of antibodies against *A. suum* cuticular collagens of different size categories allows confirming the immunogenicity of these antigens. The presence of antibodies reveals that the mice produced an immune response against cuticular collagens and that cuticular collagens are antigens that possess immunogenic properties.
Immunoblots performed with the three different antisera (a-120colA.s, a-50-70colA.s, and a-30colA.s) gave the same results except in the intensity of the bands. The immunogenicity of the cuticular collagen proteins is increased in the dimer and trimer forms.
To ensure that the produced antibodies react against cuticular collagens, an immunoblot on *A. suum* cuticular collagens was performed after a digestion with collagenase. Results showed that antisera also reacted with the digested proteins. The bands are very similar to those discussed in paragraph 3.3, and indicate a very similar protein profile.
As a conclusion, the three different antisera bind to the same epitopes independently from the fact that they came from monomer, dimer or trimer. Collagens in the form of monomer, dimer or trimer are immunogenic and the antibodies they induced, when injected into mice, react in the same way with cuticular collagen of *A. suum*.

Perhaps a natural polymerization occurred spontaneousely during the immunization procedure so that the result might represent antibodies produced against a partially polymerized immunogen.

4.3 Specificity of the produced antibodies

The use of the produced antibodies for immunoblots on *C. elegans* cuticular collagens reveals the non-specificity of the antibodies to their immunogen. The produced antibodies are not specific against *A. suum* cuticular collagens because they also react with *C. elegans* cuticular collagen. However the immunoblot results are not the same when performed with the different antisera a-120colA.s, *B. pahangi* (a-205) and CD 68. Therefore a certain degree of specificity is present.

Immunoblot on *C. elegans* cuticular collagen with antisera a-120colA.s and CD 68 reveal the presence of a band around 30 kDa that probably indicates the presence of the monomer form (Fig. 7). The presence of this band is surprinsing because no band was observed on SDS Polyacrylamid gel. The higher sensibility of the immunoblot can explain the presence of this band.

Immunoblot on *B. pahangi* cuticular proteins using different antisera reveals different profiles (unpublished results). Antisera a-120colA.s on *B. pahangi* cuticular proteins revealed several bands, which indicates that it is not specific (unpublished results). Antisera *Bpcol-1* (a-19-1) did not react with cuticular proteins of *C. elegans*. However immunoblot of *Bpcol-1* (a-19-1) on *B. pahangi* cuticular collagens were positive. This observation confirms the specificity of the antiserum *Bpcol-1* (a-19-1) for *B. phangi*. No cross-reaction is observed with *A. suum* or *C. elegans* cuticular collagens.

All these antibodies except the *Bpcol-1* (a-19-1) are not specific. As the cuticular collagens of nematodes are quite similar in their structure and show an important homology in their sequences, it is not surprising that antibodies against a specific cuticular collagen react with several cuticular collagens. On the contrary, an antiserum produced on the basis of a recombinant protein *(Bpcol-1)* was specific. A more detailed study on the specificity of the antiserum *Bpcol-1* (a-19-1) is performed in the following chapters.

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CHAPTER 3: ARE THERE IDENTICAL COLLAGEN GENES IN DIFFERENT NEMATODES ?

1 Introduction

According to the results obtained in the previous chapter, our interest was focused on the gene *Bpcol-1*, a gene that codes for *B. pahangi* cuticular collagen. The isolation and the characterization of this gene were done by Bisoffi (Bisoffi and Betschart, 1996). They determined the sequence of the *Bpcol-1* gene, after its cloning into the sequencing vector M13mp 18/19, by the dei-deoxy chain-termination method (Sanger *et al.*, 1977). They described the existence of a coding sequence of 657-bp, whose sequence analysis revealed an open reading frame encoding 218 amino acids. The calculated molecular weight of this polypeptide is 24 kDa. 156 amino acids of *Bpcol-1* represent $(Gly\text{-}X\text{-}Y)_n$ triple-helical motifs, which are typical for collagens (bolded in Table 1). The proline content within the triple-helical motifs of *Bpcol-1* is 29,5%. Those motifs are interrupted by short stretches of 4-18 amino acids that depart from a repeating $(Gly\text{-}X\text{-}Y)_n$ sequence. That protein structure has been shown to be typical for collagen proteins of the cuticle of several nematode species (Cox, 1992). However the gene *Bpcol-1* discovered by Bisoffi was not complete. Martin and coworkers isolated from *B. pahangi* a 273-bp fragment (Martin *et al.*, 1996). Their clone corresponds to the missing part of the gene *Bpcol-1*. During his manipulations, Bisoffi used the *EcoRI* restriction site for insertion into the vector M13. This site was also present inside the gene *Bpcol-1*, so that only a part of the *Bpcol-1* gene was inserted into the vector.
According to the results obtained by Martin and coworkers, the final sequence analysis of the complete *Bpcol-1* gene revealed an open reading frame coding 289 amino acids. The deduced amino acid sequence is shown in Table 1. The calculated molecular weight of this polypeptide is around 30 kDa that corresponds to the size of the monomer for cuticular collagens.
As described by Johnstone, (Johnstone, 1993), cuticular collagen genes are divided into six classes. The predicted protein sequence of *Bpcol-1* presented the organization of cysteines and of typical triple helical regions of the class B collagens (see page 20). The last nine amino acids of the carboxyl end of the C terminus were also in total agreement with those of class B collagen (Kramer, 1994) (Table 1). Among numerous genes of the class B, the three genes *Ascol-C* (Di Mito and Betschart, 1999), *Bpcol-1* (Martin *et al.*, 1996) and *Bmcol-2* (Scott *et al.*, 1995) were choosen for the studies in this chapter. These three genes belong to the three different parasite nematodes *A.suum, B. pahangi* and *B. malayi.* The choice of these three genes was made by the fact that they are genes that encode cuticular collagens of parasite nematodes and that they belong to the same class of gene and their product have a high percentage of homology in their amino acid sequences. The word “gene” is used as a general term even if it consists of the coding sequence of the gene.
Studies made by Di Mito and Betschart (1999) allow isolating and characterizing a new gene encoding cuticular collagen proteins in *A. suum*. They determined the sequence of this gene, named *Ascol-C*. They found that the overall nucleotide homology between *Ascol-C* and *Bpcol-1* was 70% and the overall amino acid homology was 77 %.
According to the results found in the previous chapter, antibodies *Bpcol-1* (a-19-1) are specific to *B. pahangi*, it will be interesting to see if the gene *Bpcol-1* can also be found in *A. suum*. This will not exclude the specificity of the *Bpcol-1* protein but will allow a better understanding of the formation of the collagen triple helix structure.
The comparison of amino acid sequences of *Ascol-C*, *Bpcol-1* and *Bmcol-2* proteins showed that, if gaps were introduced into the amino acid sequences in order to maximize the alignment, the region I, II and III (Cox, 1989) of *Ascol-C* and *Bpcol-1* were similar to those of *Bmcol-2* of *B. malayi* (Scott, 1995) (Table 1).

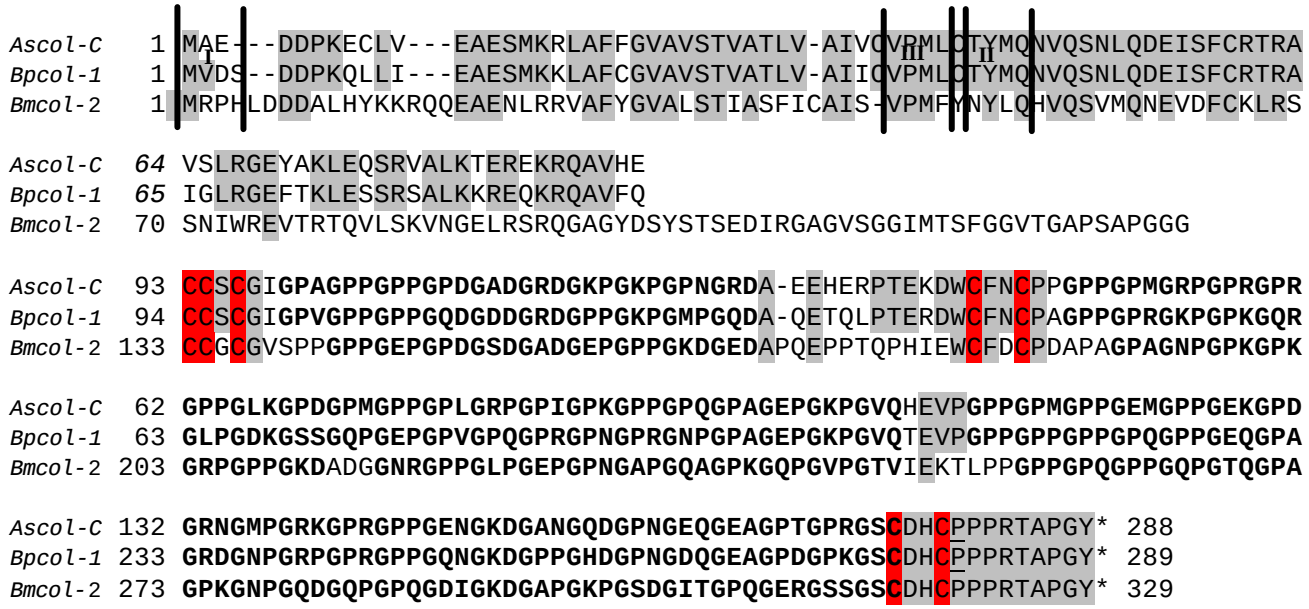

Table 1: Comparison of the amino acid sequences of different collagen proteins. The localization of the three regions I, II and III (Johnstone, 1993) is indicated by vertical lines. *Ascol-C* [Di Mito and Betschart, 1999] is compared with proteins sequences of *B. pahangi* [*Bpcol-1* (Martin *et al*., 1996)] and *B. malayi* [*Bmcol-2* (Scott *et al*., 1995)]. Gaps have been introduced to maximize alignment. Conserved cysteine residues are boxed in red color and bolded amino acids indicate triple-helical domain. Grey-coloured amino acids indicate nucleotides positioned outside of the triple helical domains that are conserved. Asterisks indicate termination codons.

Therefore it was attempted to identify identical sequences of these three genes in *A. suum* and *B. pahangi*. Since these three genes belong to the same class of collagens it would not be surprising to find common genes in different species. By common it let it be understood that nucleotides sequence are very similar, almost identical.

2 Materials and methods

2.1 Molecular biology material

2.1.1 cDNA library

The cDNA library of *Brugia pahangi* was prepared in the phage λUniZap XR from mRNA from L3 gathered 3 days after infection. The library was a kind gift of Dr. Hunter, University of Liverpool, U.K.
The cDNA library of *Ascaris suum* hypodermis was prepared in the phage λZapII. The library was a kind gift of Dr. Geary and Klein (The Upjohn Company Kalamazoo, USA).

2.1.2 Bacteria strains, vectors and enzymes

- XL1-Blue-recA$^-$ strain: *E. coli* (recA1, lac$^-$, end A1, gyrA96, thi, hsdR17, supE44, relA1, [F', proAB, lac/q, LacZΔM15, Tn10] (tet^r)). This strain was a kind gift of the Biochemistry laboratory of Neuchâtel.
  XL1-Blue *E. coli* strain was used to replicate pGEM®-T-Easy Vector (Promega) recombinant vector.

- pGEM®-T-Easy Vector (Promega). This vector is used with XL1-Blue-recA$^-$ strain.

- Restriction enzymes and buffer were purchased from Promega. T4 DNA ligase by Promega was used for ligation. XL1-blue competent cells were used for cloning. Sequencing TaqDNA polymerase and buffer were purchased from Qiagen.

2.1.3 Primers

All primers were synthesized by MWG-Biotech (Ebersberg, Deutschland).

Specific primers for vectors

pGEM®-T-Easy Vector	M13 primer	5'-gtaaaacgacggccagt-3'	T_a = 56°C
	M13 Reverse primer	5'-ggaaacagctatgaccatg-3'	T_a = 56°C

T_a corresponds to the temperature of Annealing

Specific primers for cuticular collagen genes

Bpcol-1 5' primer *Bpcol-1* 3' primer	5'-**ggatcc**atggttgattctgatgatccg- 3' 5'-**cccggg**ttaatagcctggagctgttct- 3'	T_a = 65°C T_a = 68°C
Ascol-C 5' primer *Ascol-C* 3' primer	5'-**ggatcc**atggctgaagacgatccaaagg- 3' 5'-**cccggg**ttaatatccaggtgccgttct- 3'	T_a = 68°C T_a = 68°C
Bmcol-2 5' primer *Bmcol-2* 3' primer	5'-**ggatcc**atgaggcctcacctggacgatgat- 3' 5'-**cccggg**ttaatagccaggtgctgttcgtgg- 3'	T_a = 68°C T_a = 68°C

Bold characters correspond to restriction sites.

Bpcol-1 5' and 3' correspond to the flanking region of the collagen gene *Bpcol-1* preceded respectively by the restriction sites *BamHI* and *XmaI*. *Ascol-C* 5' and 3' primers and *Bmcol-2* 5' and 3' primers correspond respectively to the flanking region of the collagen gene *Ascol-C* and *Bmcol-2* preceded by the restriction sites *BamHI* and *XmaI*.

2.1.4 Sequencing

Sequencing was made with Amersham Thermo sequenase primer cycle sequencing kit and with an automatic sequencer Li-Cor 4000L. All the samples were sequenced 4 times.
Primers were M13 and M13 Reverse for a sequence inside pGEM®-T-Easy Vector. These primers are already modified in 5' by IRD 800 chromophore. The analyses of sequences were carried out with a free Internet software. http://www.searchlauncher.bcm.tmc.edu/multi-align/multi-align.html

2.2 Molecular biology methods

2.2.1 Digestion and purification of DNA

The digestion was made in a final volume of 20µl following the manufacturer's instructions at 37°C for three hours. DNA fragments provided from digestion (or PCR amplification) were separated by electrophoresis on 0,7 % agarose gel. The recovery of the fragments was made by directly pipeting the bands from the agarose gel.

2.2.2 Ligation

As the digested, purified vectors and inserts are recovered directly by pipeting the bands from the agarose gel, it is difficult to give the exact quantity of DNA used for the ligation. It was tried to use a proportion of 1:2 of vector and insert.
The ligation is performed in a final volume of 15 µl following the manufacturer's instruction overnight at 14°C.

2.2.3 Transformation

150 µl of competent cells were mixed with 2/3 of the ligation product and incubated in ice during 30 minutes. After 2 minutes of heat shock at 42°C, the cells were incubated in ice during 5 minutes and plated on solid LB-Agar medium with selective antibiotics and, finally, incubated overnight at 37°C. The selected colonies were tested by PCR with the appropriate primers:

DNA template (colony)	Bacteria resuspended in water
5' primer	3µM
3' primer	3µM
Nucleotide tri-phosphates	200µM
Reaction buffer	10X
TaqDNA polymerase	1 Unit
Reaction volume	30µl

PCR program

1)	95°C	1min	Initial denaturation	
2)	95°C	30 sec	Denaturation	
3)	T_a°C	30 sec	Annealing	
4)	72°C	1min	Elongation	Steps 2-4 (30 cycles)
5)	72°C	10min		
6)	10°C			

Remarks: If the insertion takes place inside the lacZ part of the vector, it is possible to make a white-blue selection of the colonies. This selection is possible by adding X-Gal and IPTG on solid LB-Agar medium. The selected white colonies are the transformed colonies. However it is more secure to test by PCR these white colonies.

2.2.4 Selection of the coding sequence of the gene *Bpcol-1*

Before proceeding to the PCR of the cDNA library, it is necessary to precipitate the cDNA with an aqueous solution of sodium acetate and a solution of EtOH 100%. The amplification of the coding sequence of the gene *Bpcol-1*, *Ascol-C* and *Bmcol-2* from the cDNA library of *B. pahangi* and *A. suum* were done by PCR. To isolate these different coding sequences the primers described on page 45 were used.

cDNA precipitated	5µl
5' primer	5µM
3' primer	5µM
Nucleotide tri-phosphates	200µM
Reaction buffer	10X
TaqDNA polymerase	1 Unit
Reaction volume	50µl

PCR program				
1)	95°C	10min	Initial denaturation	
2)	95°C	30 sec	Denaturation	
3)	T_a	30 sec	Annealing	
4)	72°C	1min	Elongation	Steps 2-4 (35 cycles)
5)	72°C	5min		
6)	10°C			

3 Results

3.1 Amplification and sequence analysis of class B cuticular collagen genes

Using *Ascol-C* 5' and 3' primers an analogue of the gene *Ascol-C* was amplified by PCR using directly a cDNA library of *B. pahangi.* The analysis on agarose gel of the PCR results shows a band around 900-bp corresponding to the size of the gene *Ascol-C* (Di Mito and Betschart, 1999) (Fig. 1).
Using *Bpcol-1* 5' and 3' primers an analogue of the gene *Bpcol-1* was amplified by PCR using directly a cDNA library of *A. suum*. The analysis on agarose gel of the PCR results shows a band around 900-bp corresponding to the size of the gene *Bpcol-1* (Martin *et al*., 1996) (Fig. 1).
Using *Bmcol-2* 5' and 3' primers an analogue of the gene *Bmcol-2* was amplified by PCR using directly a cDNA library of *B. pahangi.* The analysis on agarose gel of the PCR results shows a band around 900-bp corresponding to the size of the gene *Bmcol-2* (Scott *et al*., 1995) (Fig. 1).
No result appears when *Bmcol-2* 5' and 3' primers were used to try to amplify by PCR an analogue using directly a cDNA library of *A. suum*.
PCR amplification on cDNA library of *B. malayi* was not possible because the library was not available. Therefore it was not possible to do all combinations (Table 2)
The purification of the 900-bp fragments and their ligation into the vector pGEM®-T-Easy vector was performed. After the transformation of XL1-Blue cells, a selection of white colonies was checked by PCR with M13 and M13 reverse primers. Agarose gel analysis of the PCR shows bands around 1000-bp that correspond to the size of the gene plus a part of the pGEM®-T-Easy vector (Fig. 2). The nature of the clones was verified by digestion with the enzymes *BamHI* and *XmaI* (Fig. 3-4) and sequenced four times (Table 3). In the three cases, the agarose gel analysis of the single digestion with *BamHI* gives a band of 3900-bp corresponding to the size of the pGEM®-T-Easy Vector (3000-bp) plus the inserted gene (900-bp). The double digestion shows one band of 900-bp that corresponds to the size of the gene and one band of 3000-bp that corresponds to the size of the vector.

	Ascol-C 5' and 3' primers	*Bpcol-1* 5' and 3' primers	*Bmcol-2* 5' and 3' primers
cDNA of *Ascol-C*	yes / positive result	yes / positive result	yes / negative result
cDNA of *Bpcol-1*	yes / positive result	yes / positive result	yes / positive result
cDNA of *Bmcol-2*	not available	not available	not available

Table 2: Representation of the different combinations of PCR amplification done on different cDNA libraries.

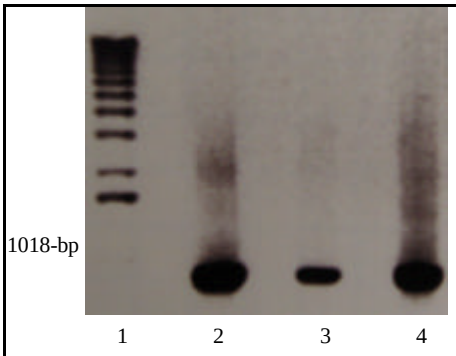

Fig. 1: **PCR**: lane 1: DNA ladder; lane 2: Analogous *Bpcol-1* gene obtained by PCR with *Bpcol-1* 5' and 3' primers on *A. suum* cDNA, lane 3: Analogous *Ascol-C* gene amplified with *Ascol-C* 5' and 3' primers on *B. pahangi* cDNA, lane 4: Analogous *Bmcol-2* gene amplified with *Bmcol-2* 5' and 3' primers on *B. pahangi* cDNA.

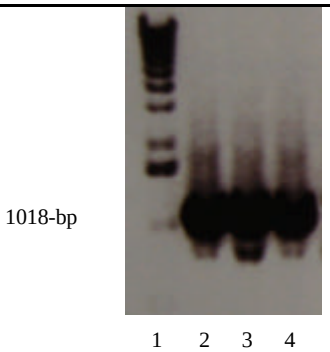

Fig. 2: **PCR**: lane 1: DNA ladder; lane 2: Analogous *Bpcol-1* gene obtained by PCR amplification with M13 and M13 reverse on white transformed colonies, lane 3: Analogous *Ascol-C* gene obtained by PCR amplification with M13 and M13 reverse on white transformed colonies, lane 4: Analogous *Bmcol-2* gene obtained by PCR amplification with M13 and M13 reverse on white transformed colonies.

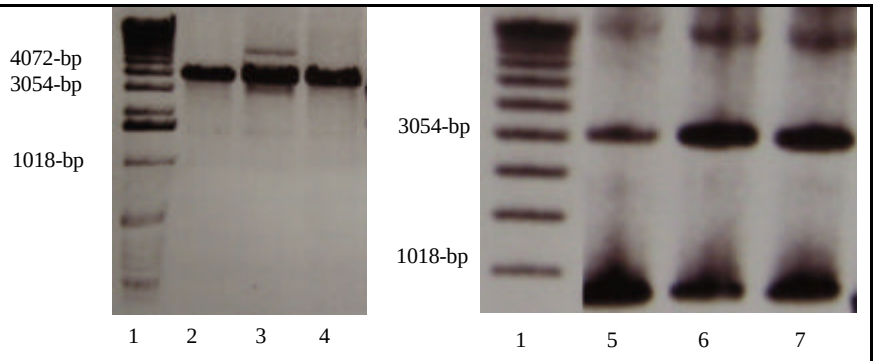

Fig. 3-4: **Digestion**: lane 1: DNA ladder; lane 2: pGEM® + analogous *Bpcol-1* digested with *BamHI*; lane 5: pGEM® + analogous *Bpcol-1* digested with *BamHI* and *XmaI*; lane 3: pGEM® + analogous *Ascol-C* digested with *BamHI*; lane 6: pGEM® + analogous *Ascol-C* digested with *BamHI* and *XmaI*; lane 4: pGEM® + analogous *Bmcol-2* digested with *BamHI*; lane 7: pGEM® + analogous *Bmcol-2* digested with *BamHI* and *XmaI.*

A. (using primers *Ascol-C* 5' and 3')

```
Ascol-C c/o B.pahangi   1 CATGGCGGCCGCGGGAATTCGATTGGATCCATGGCTGAAGACGATCCAAAGGAGTGTCTC
Ascol-C                 1 ------GG----A--A----AGTT-GA---ATGGCTGAAGACGATCCAAAGGAGTGTCTC
consensus               1 ------**-------*------**-**---*****************************

Ascol-C c/o B.pahangi  61 GTCGAGGCCGAGAGCATGAAGAGGCTCGCCTTCTTCGGCGTCGCCGTATCAACTGTGGCT
Ascol-C                52 GTCGAGGCCGAGAGCATGAAGAGGCTCGCCTTCTTCGGCGTCGCCGTATCAACTGTAGCT
consensus              61 ************************************************************_***

Ascol-C c/o B.pahangi 121 ACGTTGGTTGCCATCGTTTGCGTGCCAATGCTCTGCACCTATATGCAAAATGTGCAGTCG
Ascol-C               111 ACGTTGGTTGCCATCGTTTGCGTGCCAATGCTCTGCACCTATATGCAAAATGTGCAGTCG
consensus             121 ************************************************************

Ascol-C c/o B.pahangi 181 AATCTCCAGGACGAGATCAGTTTCTGCCGAACCCGCGCAGTCTCTCTTCGCGGAGAGTAT
Ascol-C               171 AATCTCCAGGACGAGATCAGTTTCTGCCGAACCCGCGCAGTCTCTCTTCGCGGAGAGTAT
consensus             181 ************************************************************

Ascol-C c/o B.pahangi 241 GCAAAGCTGGAACAATCTCGTGTCGCACTCAAAACAGAAAGAGAGAAGCGCCAGGCGGTG
Ascol-C               231 GCAAAGCTGGAACAATCTCGTGTCGCACTCAAAACAGAAAGAGAGAAGCGCCAGGCGGTG
consensus             241 ************************************************************

Ascol-C c/o B.pahangi 301 CATGAATGCTGCAGCTGCGGTATTGGTCCAGCTGGACCGCCAGGTCCACCGGGACCTGAT
Ascol-C               291 CATGAATGCTGCAGCTGCGGTATTGGTCCAGCTGGACCGCCAGGTCCACCGGGACCTGAT
consensus             301 ************************************************************

Ascol-C c/o B.pahangi 361 GGCGCAGACGGCCGCGACGGCAAGCCAGGGAAACCTGGTCCAAACGGTCGTGACGCTGAG
Ascol-C               351 GGCGCAGACGGCCGCGACGGCAAGCCAGGGAAACCTGGTCCAAACGGTCGTGACGCTGAG
consensus             361 ************************************************************

Ascol-C c/o B.pahangi 421 GAACATGAGAGACCAACAGAGAAGGACTGGTGCTTCAACTGCCCGCCTGGACCACCAGGA
Ascol-C               411 GAACATGAGGGACCAACAGAGAAGGACTGGTGCTTCAACTGCCCGCCTGGACCACCAGGA
consensus             421 *********_**********************************************

Ascol-C c/o B.pahangi 481 CCCATGGGACGACCAGGACCACGAGGACCTCGTGGACCTCCAGGACTCAAAGGTCCCGAT
Ascol-C               470 CCCATGGGACGACCAGGACCACGAGGACCTCGTGGACCTCCAGGACTCAAAGGTCCCGAT
consensus             481 ************************************************************

Ascol-C c/o B.pahangi 541 GGGCCAATGGGACCTCCAGGACCACTGGGACGACCAGGCCCAATTGGACCAAAAGGACCT
Ascol-C               530 GGGCCAATGGGACCTCCAGGACCACTGGGACGACCAGGCCCAATTGGACCAAAAGGACCT
consensus             541 ************************************************************

Ascol-C c/o B.pahangi 601 CCGGGACCTCAAGGACCTGCTGGCGAACCAGGCAAGCCCGGTGTGCAGCATGAGGTTCCT
Ascol-C               590 CCGGGACCTCAAGGACCTGCTGGCGAACCAGGCAAGCCCGGTGTGCAGCATGAGGTTCCT
consensus             601 ************************************************************

Ascol-C c/o B.pahangi 661 GGACCACCAGGACCAATGGGACCTCCTGGAGAGATGGGACCTCCAGGCGAGAAAGGGCCT
Ascol-C               650 GGACCACCAGGACCAATGGGACCTCCTGGAGAGATGGGACCTCCAGGCGAGAAAGGGCCT
consensus             661 ************************************************************

Ascol-C c/o B.pahangi 721 GACGGACGTAATGGCATGCCAGGCCGCAAGGGTCCGCGTGGTCCTCCAGGCGAAAATGGC
Ascol-C               710 GACGGACGTAATGGCATGCCAGGCCGCAAGGGTCCGCGTGGTCCTCCAGGCGAAAATGGC
consensus             721 ************************************************************

Ascol-C c/o B.pahangi 781 AAGGACGGAGCCAACGGCCAAGATGGTCCAAATGGCGAACAAGGAGAGGCTGGCCCAACG
Ascol-C               770 AAGGACGGAGCCAACGGCCAAGATGGTCCAAATGGCGAACAAGGAGAGGCTGGCCCAACG
consensus             781 ************************************************************

Ascol-C c/o B.pahangi 841 GGTCCAAGAGGAAGTTGCGACCACTGCCCGCCGCCCAGAACGGCACCTGGATATTAACCC
Ascol-C               830 GGTCCAAGAGGAAGTTGCGACCACTGCCCGCCGCCCAGAACGGCACCTGGATATTAA---
consensus             841 ************************************************************---

Ascol-C c/o B.pahangi 901 GGGAATCACTAGTGA-----ATTCGCGGCCGCCTGCAGGTCGAC--------CATATGGG
Ascol-C               890 ---A-T-G---GTGA-----ATT---GACCGCCTG-AGATCG----------CAT--G--
consensus             901 ---*-*-----****-----***---*-*******-**-***----------***--*--

Ascol-C c/o B.pahangi 948 AGAGCTCCCAACGCGTT--GGATGCATAGCTTGAGTATTCTATAGTGTCA----------
Ascol-C               947 A-A--T-CCGA-G--TT--GG-T---TA--T--A-T--T--A---T-TC----------
consensus             961 *-*--*-**-*-*--**--**-*---**--*--*-*--*--*---*-**---------
```

B. (using primers *Bpcol-1* 5' and 3')

```
Bpcol-1 c/o A.suum      1 CATGGCGGCCGCGGGAATTCGATTGGATCCATGGTTGATTCTGATGATCCGAAACAGCTT
Bpcol-1                 1 -GT---A----C-AAG-TT-AG-TAG----ATGGTTGATTCTGATGATCCGAAACAGCTT
consensus               1 --*--------*-----**----*-*----****************************

Bpcol-1 c/o A.suum     61 CTCATTGAGGCAGAAAGCATGAAAAAGCTTGCCTTTTGCGGTGTTGCTGTTTCTACCGTA
Bpcol-1                53 CTCATTGAGGCGGAAAGCATGAAAAAGCTTGCCTTTTGCGGTGTTGCTGTTTCTACCGTA
consensus              61 **********-**************************************************

Bpcol-1 c/o A.suum    121 GCTACTCTGGTAGCAATTATTTGCGTACCAATGCTCTGCACCTACATGCAAAATGTGCAG
Bpcol-1               112 GCTACTCTGGTAGCAATTATTTGCGTACCAATGCTCTGCACCTACATGCAAAATGTGCAG
consensus             121 ************************************************************

Bpcol-1 c/o A.suum    181 TCTAACTTGCAAGATGAGATTAGCTTCTGCAGGACCCGCGCAATTGGATTACGAGGAGAA
Bpcol-1               172 TCTAACTTGCAAGATGAGATTAGCTTCTGCAGGACACGCGCAATTGGATTACGAGGAGAA
consensus             181 ***********************************-************************

Bpcol-1 c/o A.suum    241 TTCACCAAACTCGAATCATCGCGTTCAGCACTGAAAAAAGAAAGACAAAAGAGGCAGGCA
Bpcol-1               232 TTCACCAAACTCGAATCATCGCGTTCAGCACTGAAAAAAGAAAGACAAAAGAGGCAGGCA
consensus             241 ************************************************************

Bpcol-1 c/o A.suum    301 GTATTCCAATGTTGCAGTTGCGGTATTGGTCCTGTTGGTCCACCAGGCCCACCTGGACAA
Bpcol-1               292 GTATTCCTATGTTGCAGTTGCGGTATTGGTCCTGTTGGTCCACCAGGCCCACCTGGACAA
consensus             301 *******-****************************************************

Bpcol-1 c/o A.suum    361 GATGGTGACGATGGCCGAGATGGCCCACCTGGAAAACCTGGTATGCCAGGTCAGGATGCT
Bpcol-1               352 GATGGTGACGATGGCCGAGATGGCCCACCTGGAAAACCTGGTATGCCAGGTCAGGATGCT
consensus             361 ************************************************************

Bpcol-1 c/o A.suum    421 CAAGAAACCCAACTGCCAACTGAACGAGACTGGTGTTTCAATTGTCCTGCAGGACCACCA
Bpcol-1               412 CAAGAAACCCAACTGCCAACTGAACGAGACTGGTGTTTCAATTGTCCTGCAGGACCACCA
consensus             421 ************************************************************

Bpcol-1 c/o A.suum    481 GGTCCACGAGGTAAACCAGGACCAAAAGGACAGAGAGGATTGCCGGGAGACAAAGGTTCT
Bpcol-1               472 GGTCCACGAGGTAAACCAGGACCAAAAGGACAGAGAGGATTGCCGGGAGACAAAGGTTCT
consensus             481 ************************************************************

Bpcol-1 c/o A.suum    541 AGCGGACAGCCTGGTGAACCGGGTCCTGTGGGACCACAAGGGCCAAGAGGACCGAATGGA
Bpcol-1               532 AGCGGACAGCCTGGTGAACCGGGTCCTGCGGGACCACAAGGGCCAAGAGGACCGAATGGA
consensus             541 ****************************-***********************************

Bpcol-1 c/o A.suum    601 CCTCGAGGAAATCCAGGCCCAGCTGGCGAACCAGGAAAACCGGGTGTACAGACTGAGGTG
Bpcol-1               592 CCTCGAGGAAATCCAGGCCCAGCTGGCGAACCAGGAAAACCGGGTGTACAGACTGAGGTG
consensus             601 ************************************************************

Bpcol-1 c/o A.suum    661 CCTGGACCACCAGGACCACCTGGCCCACCAGGACCACAAGGACCACCAGGTGAGCAAGGT
Bpcol-1               652 CCTGGACCACCAGGACCACCTGGCCCACCAGGACCACAAGGACCACCAGGTGAGCAAGGT
consensus             661 ************************************************************

Bpcol-1 c/o A.suum    721 CCAGCTGGGCGAGATGGCAATCCCGGACGACCAGGACCACGTGGACCACCAGGTCAGAAC
Bpcol-1               712 CCAGCTGGGCGAGATGGCAATCCCGGACGACCAGGACCACGTGGACCACCAGGTCAGAAC
consensus             721 ************************************************************

Bpcol-1 c/o A.suum    781 GGCAAAGATGGTCCTCCAGGACATGATGGTCCAAATGGTGATCAAGGAGAAGCAGGTCCC
Bpcol-1               772 GGCAAAGATGGTCCTCCAGGACATGATGGTCCAAATGGTGATCAAGGAGAAGCAGGTCCC
consensus             781 ************************************************************

Bpcol-1 c/o A.suum    841 GATGGACCAAAAGGCAGTTGTGACCATTGTCCACCTCCCAGAACAGCTCCAGGCTATTAA
Bpcol-1               832 GATGGACCAAAAGGCAGTTGTGACCATTGTCCACCTCCCAGAACAGCTCCAGGCTATTAA
consensus             841 ************************************************************

Bpcol-1 c/o A.suum    901 CCCGGGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGA
Bpcol-1               892 -C-GGGA-T--C-AG-AGA-T-A-----ACCTG--A-TCA--C---TAG-A--
consensus             901 -*-****-*--*-**---*-*--------****----**---*---*-*-*--
```

C. (using primers *Bmcol-2* 5' and 3')

```
Bmcol-2 c/o B.pahangi     1 GGCCGCGGGAATTCGATTGGATCCATGAGGCCTCACCTGGACGATGATGCATTACATTAT
Bmcol-2                   1 GGTTTAATTACCCAAGTTTGAGTGATGAGGCCTCACCTGGACGATGATGCATTACATTAT
consensus                 1 **-------*------**-**---**********************************

Bmcol-2 c/o B.pahangi    61 AAAAAAAGACAACAGGAAGCGGAAAATCTTCGAAGGGTTGCATTTTACGGTGTAGCATTA
Bmcol-2                  58 AAAAAAAGACAACAGGAAGCGGAAAATCTTCGAAGGGTTGCATTTTACGGTGTAGCATTA
consensus                61 ************************************************************

Bmcol-2 c/o B.pahangi   121 TCAACCATAGCTTCATTTATATGCGCAATTTCCGTACCAATGTTTTATAATTATTTGCAA
Bmpcol-2                118 TCAACCATAGCTTCATTTATATGCGCAATTTCCGTACCAATGTTTTATAATTATTTGCAA
consensus               121 ************************************************************

Bmcol-2 c/o B.pahangi   181 CATGTACAATCTGTTATGCAAAATGAAGTGGACTTTTGTAAATTACGCTCAAGTAATATT
Bmcol-2                 178 CATGTACAATCTGTTATGCAAAATGAAGTGGACTTTTGTAAATCACGCTCAAGTAATATT
consensus               181 ******************************************-*****************

Bmcol-2 c/o B.pahangi   241 TGGCGTGAAGTAACACGAACACAGGTATTATCAAAAGTAAATGGTGAATTACGGAGCCGT
Bmcol-2                 238 TGGCGTGAAGTAACACGAACACAGGTATTATCAAAAGTAAATGGTGAATTACGGAGCCGT
consensus               241 ************************************************************

Bmcol-2 c/o B.pahangi   301 CAAGGAGCCGGTTATGACAGCTATTCGACCAGTGAGGACATACGTGGTGCTGGTGTCAGT
Bmcol-2                 298 AAAGGAGCCGGTTATGACAGCTATTCGACCAGTGAGGACATACGTGGTGCTGGTGTTAGT
consensus               301 -************************************************-***********

Bmcol-2 c/o B.pahangi   361 GGTGGTATTATGACAAGTTTTGGTGGAGTTACGGGTGCACCTTCTGCACCTGGTGGCGGT
Bmcol-2                 358 GGTAGTGTTATGACAAGTTTTGGTGGAGTTACGGGTGCACCTTCTGCACCTGGTGGTGGT
consensus               361 ***-**-************************************************-***

Bmcol-2 c/o B.pahangi   421 TGCTGCGGTTGTGGTGTATCACCACCTGGGCCACCTGGTGAGCCTGGTCCAGATGGAAGT
Bmcol-2                 416 TGCTGCGGTTGTGGTGTATCACCACCTGGGCCACCTGGTGAGCCTGGTCCAGATGGAAGT
consensus               421 ************************************************************

Bmcol-2 c/o B.pahangi   481 GATGGTGCTGATGGTGAACCAGGACCACCAGGTAAAGATGGAGAGGATGCACCACAGGAA
Bmcol-2                 476 GATGGTGCTGATGGTGAACCAGGACCACCAGGTAAAGATGGAGAGGATGCACCACAGGAA
consensus               481 ************************************************************

Bmcol-2 c/o B.pahangi   541 CCACCTACGCAACCTCATATTGAATGGTGTTTCGATTGTCCAGATGCACCAGCCGGTCCA
Bmcol-2                 536 CCACCTACGCAACCTCATATTGAATGGTGTTTCGATTGTCCAGATGCACCAGCCGGTCCA
consensus               541 ************************************************************

Bmcol-2 c/o B.pahangi   601 GCAGGAAATCCAGGACCGAAAGGTCCAAAGGGTAGACCTGGACCACCAGGAAAGGATGCA
Bmcol-2                 596 GCAGGAAATCCAGGACCGAAAGGTCCAAAGGGTAGACCTGGACCACCAGGAAAGGATGCA
consensus               601 ************************************************************

Bmcol-2 c/o B.pahangi   661 GATGGTGGTAATAGAGGACCACCAGGACTGCCAGGTGAACCAGGACCAAATGGCGCACCA
Bmcol-2                 656 GATGGTGGTAATAGAGGACCACCAGGACCGCCAGGTGAACCAGGACCAATTGGCGCACCA
consensus               661 ****************************-******************-**********

Bmcol-2 c/o B.pahangi   721 GGTCAAGCAGGTCCAAAAGGACAACCAGGAGTGCCTGGAACTGTGATTGAAAAAACTCTA
Bmcol-2                 716 GGTCAAGCAGGTCCAAAAGGACAACCAGGAGTGCCTGGAACTGTGATTGAAAAAACTCTA
consensus               721 ************************************************************

Bmcol-2 c/o B.pahangi   781 CCACCAGGTCCACCAGGACCACAAGGGCCACCTGGTCAACCCGGTACACAAGGACCAGCT
Bmcol-2                 776 CCACCAGGTCCACCAGGACCACAAGGGCCACCTGGTCAACCCGGTACACAAGGACCAGCT
consensus               781 ************************************************************

Bmcol-2 c/o B.pahangi   841 GGACCGAAAGGTAATCCTGGACAAGATGGACAACCAGGACCACAAGGTGATATAGGAAAA
Bmcol-2                 836 GGACCGAAAGGTAATCCTGGACAAGATGGACAACCAGGACCACAAGGTGATATAGGAAAA
consensus               841 ************************************************************

Bmcol-2 c/o B.pahangi   901 GATGGAGCACCTGGTAAACCAGGTAGCGATGGAATAACTGGACCACAAGGAGAACGTGGT
Bmcol-2                 896 GAAGGAGCACCTGGTAAACCAGGCAGCGATGGAATAACTGGACCACAAGGAGAACGTGGT
consensus               901 **-********************-************************************

Bmcol-2 c/o B.pahangi   961 TCTTCCGGTTCATGCGATCATTGTCCACCACCACGAACAGCACCTGGCTATTAACCCGGG
Bmcol-2                 956 TCTTCCGGTTCATGCGATCATTGTCCACCACCACGAACAGCATTTGGCTATTAACACATA
consensus               961 ****************************************__**********-*---
```

```
Bmcol-2 c/o B.pahangi   1021 AATCACTAGTGAATTCGCGGCCGCCTG
Bmcol-2                 1014 TCATTTGATATAATATGCTACACCGAT
consensus               1021 -------*---***--**--*--*---
```

Table 3: **(A)** Sequence alignment of *Ascol-C* gene (Di Mito and Betschart, 1999) with the analogous gene *Ascol-C* isolated from *B. pahangi* cDNA library. **(B)** Sequence alignment of *Bpcol-1* gene (Martin, S.A. *et al*., 1996) with the analogous gene *Bpcol-1* isolated from *A. suum* cDNA library. **(C)** Sequence alignment of *Bmcol-2* gene (Scott, *et al*., 1995) with the analogous gene *Bmcol-2* isolated from *B. pahangi* cDNA library. Shaded characters correspond to restriction sites, bolded character correspond to start and stop codons, blue chararters correspond to sequence primer and red characters correspond to differences in nucleotides sequence.

A.

```
Ascol-C c/o B.pahangi   MAEDDPKECLVEAESMKRLAFFGVAVSTVATLVAIVCVPMLCTYMQNVQS
Ascol-C                 MAEDDPKECLVEAESMKRLAFFGVAVSTVATLVAIVCVPMLCTYMQNVQS

Ascol-C c/o B.pahangi   NLQDEISFCRTRAVSLRGEYAKLEQSRVALKTEREKRQAVHECCSCGIGP
Ascol-C                 NLQDEISFCRTRAVSLRGEYAKLEQSRVALKTEREKRQAVHECCSCGIGP

Ascol-C c/o B.pahangi   AGPPGPPGPDGADGRDGKPGKPGPNGRDAEEHEGPTEKDWCFNCPPGPPG
Ascol-C                 AGPPGPPGPDGADGRDGKPGKPGPNGRDAEEHERPTEKDWCFNCPPGPPG

Ascol-C c/o B.pahangi   PMGRPGPRGPRGPPGLKGPDGPMGPPGPLGRPGPIGPKGPPGPQGPAGEP
Ascol-C                 PMGRPGPRGPRGPPGLKGPDGPMGPPGPLGRPGPIGPKGPPGPQGPAGEP

Ascol-C c/o B.pahangi   GKPGVQHEVPGPPGPMGPPGEMGPPGEKGPDGRNGMPGRKGPRGPPGENG
Ascol-C                 GKPGVQHEVPGPPGPMGPPGEMGPPGEKGPDGRNGMPGRKGPRGPPGENG

Ascol-C c/o B.pahangi   KDGANGQDGPNGEQGEAGPTGPRGSCDHCPPPRTAPGY*
Ascol-C                 KDGANGQDGPNGEQGEAGPTGPRGSCDHCPPPRTAPGY*
```

B.

```
Bpcol-1 c/o A.suum      MVDSDDPKQLLIEAESMKKLAFCGVAVSTVATLVAIICVPMLCTYMQNVQ
Bpcol-1                 MVDSDDPKQLLIEAESMKKLAFCGVAVSTVATLVAIICVPMLCTYMQNVQ

Bpcol-1 c/o A.suum      SNLQDEISFCRTRAIGLRGEFTKLESSRSALKKERQKRQAVFLCCSCGIG
Bpcol-1                 SNLQDEISFCRTRAIGLRGEFTKLESSRSALKKERQKRQAVFQCCSCGIG

Bpcol-1 c/o A.suum      PVGPPGPPGQDGDDGRDGPPGKPGMPGQDAQETQLPTERDWCFNCPAGPP
Bpcol-1                 PVGPPGPPGQDGDDGRDGPPGKPGMPGQDAQETQLPTERDWCFNCPAGPP

Bpcol-1 c/o A.suum      GPRGKPGPKGQRGLPGDKGSSGQPGEPGPAGPQGPRGPNGPRGNPGPAGE
Bpcol-1                 GPRGKPGPKGQRGLPGDKGSSGQPGEPGPVGPQGPRGPNGPRGNPGPAGE

Bpcol-1 c/o A.suum      PGKPGVQTEVPGPPGPPGPPGPQGPPGEQGPAGRDGNPGRPGPRGPPGQN
Bpcol-1                 PGKPGVQTEVPGPPGPPGPPGPQGPPGEQGPAGRDGNPGRPGPRGPPGQN

Bpcol-1 c/o A.suum      GKDGPPGHDGPNGDQGEAGPDGPKGSCDHCPPPRTAPGY*
Bpcol-1                 GKDGPPGHDGPNGDQGEAGPDGPKGSCDHCPPPRTAPGY*
```

C.

```
Bmcol-2 c/o B.pahangi   MRPHLDDDALHYKKRQQEAENLRRVAFYGVALSTIASFICAISVPMFYNY
Bmcol-2                 MRPHLDDDALHYKKRQQEAENLRRVAFYGVALSTIASFICAISVPMFYNY

Bmcol-2 c/o B.pahangi   LQHVQSVMQNEVDFCKSRSSNIWREVTRTQVLSKVNGELRSRKGAGYDSY
Bmcol-2                 LQHVQSVMQNEVDFCKLRSSNIWREVTRTQVLSKVNGELRSRQGAGYDSY
```

```
Bmcol-2 c/o B.pahangi    STSEDIRGAGVSGSVMTSFGGVTGAPSAPGGGCCGCGVSPPGPPGEPGPD
Bmcol-2                  STSEDIRGAGVSGGIMTSFGGVTGAPSAPGGGCCGCGVSPPGPPGEPGPD

Bmcol-2 c/o B.pahangi    GSDGADGEPGPPGKDGEDAPQEPPTQPHIEWCFDCPDAPAGPAGNPGPKG
Bmcol-2                  GSDGADGEPGPPGKDGEDAPQEPPTQPHIEWCFDCPDAPAGPAGNPGPKG

Bmcol-2 c/o B.pahangi    PKGRPGPPGKDADGGNRGPPGPPGEPGPIGAPGQAGPKGQPGVPGTVIEK
Bmcol-2                  PKGRPGPPGKDADGGNRGPPGLPGEPGPNGAPGQAGPKGQPGVPGTVIEK


Bmcol-2 c/o B.pahangi    TLPPGPPGPQGPPGQPGTQGPAGPKGNPGQDGQPGPQGDIGKEGAPGKPG
Bmcol-2                  TLPPGPPGPQGPPGQPGTQGPAGPKGNPGQDGQPGPQGDIGKDGAPGKPG

Bmcol-2 c/o B.pahangi    SDGITGPQGERGSSGSCDHCPPPRTAFGY*
Bmcol-2                  SDGITGPQGERGSSGSCDHCPPPRTAPGY*
```

Table 4: **(A)** Amino acids sequence alignment of *Ascol-C* protein (Di Mito and Betschart, 1999) with the analogous protein *Ascol-C* isolated in *B. pahangi*. **(B)** Amino acids sequence alignment of *Bpcol-1* protein (Martin, S.A. *et al*., 1996) with the analogous protein *Bpcol-1* isolated in *A. suum.* **(C)** Amino acids sequence alignment of *Bmcol-2* protein (Scott, *et al*., 1995) with the analogous protein *Bmcol-2* isolated in *B. pahangi*.
Gray boxed characters correspond to differences in amino acids sequence between the proteins. Conserved cysteine residues are boxed in red color and bolded amino acids indicate triple-helical domain. Asterisks indicate termination codons.

4 Discussion

The comparison of the gene amplified from *B. pahangi* cDNA with the *Ascol-C* sequence shows 2 differences in the nucleotides sequence resulting in the modification of 1 amino acid (Table 4). This result can be interpreted in two ways. Either the cDNA library of *B. pahangi* was contaminated with *A.suum* cDNA or the gene *Ascol-C* is not specific to *A. suum* species but also present in *B. pahangi* species.
The comparison of the gene amplified from *A. suum* cDNA with the *Bpcol-1* sequence shows 4 differences in nucleotides sequence resulting in the modification of 2 amino acids (Table 4). This result can also be interpreted as a contamination of the cDNA library or as the presence of the gene *Bpcol-1* in *A. suum* species.
As cuticular collagens of nematodes are quite similar in their structure and show an important homology in their amino acids sequences, it would not be surprising to find genes conserved in different species especially if these genes belong to the same class of genes, as it is the case for *Ascol-C* and *Bpcol-1*. However, dealing with species present in 2 different families, more mutations should appear between these two genes. Because of the evolution and the mutations associated, the probability to find a fully conserved gene in 2 species that belong to different families is weak. Finding analogue genes is possible but common genes would be surprising. Therefore, a contamination of the cDNA libraries should not be excluded. To validate these results a supplementary experiment should be done on new cDNA libraries or on genomic DNA. Moreover, it would be judicious to use degenerated primer in order to take the formation of codons issued from 3 different bases into account. The possibilities to find genes encoding common proteins do not imply the nucleotides sequences to be similar. The use of degenerated primer will lead to the isolation of all combinations of genes encoding a common protein.
The comparison between the *Bmcol-2* sequence and the gene amplified from *B. pahangi* cDNA with *Bmcol-2* primer reveals 12 differences resulting in the modification of 8 amino acids (Table 4). The possibility of finding a common gene between these two species is less improbable because *B. pahangi* and *B. malayi* belong to the same family and the same genus. Therefore the presence of common genes inside a family is less surprising than the presence of common genes in two different families like *A. suum and B. pahangi.* Moreover, the fact that it was impossible to amplify an analogue *Bmcol-2* gene from *A. suum* cDNA supports the hypothesis of a contamination of *A. suum* and *B. pahangi* cDNA. A contamination of *A. suum* cDNA with *Bmcol-2* was not possible because the cDNA of *B. malayi* was never present in the laboratory.
Results obtained in this chapter do not allow us to assume the presence of a common gene of *Bpcol-1* in *A. suum.* The presence of a common gene of *Ascol-C* in *B. pahangi* could not be affirmed either. It therefore remains impossible to give another explanation to the results obtained concerning the specificity of the *Bpcol-1* antibodies (a-19-1).
Due to the fact that the sequences were verified four times on individual PCR products it can be concluded that technical errors are not involved in the production of small differences between the sequences.

5 References

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CHAPTER 4: PRODUCTION AND ANALYSIS OF *BRUGIA PAHANGI* CUTICULAR COLLAGEN PROTEINS ENCODED BY *Bpcol-1*

1 Introduction

Di Mito tried to produce the recombinant protein encoded by the *Bpcol-1* gene, however the induction of the protein as well as the immunization of Balb/c mice with the supposed recombinant protein gave no result. In parallel she discovered two new genes encoding cuticular collagen proteins in *A. suum*. Considering an important sequence analogy with the *Bpcol-1* gene, she prematurely concludes: *"A study on the specificity of the response against Bpcol-1 was outdated"*. She argues: *"that the ascaridiase is a very widespread parasitose in tropical and subtropical regions, and also all around the world. In an endemic area, a strong proportion of the population has been in contact with the parasite and therefore might have produced antibodies against Ascaris. A test based on the protein encoded by the Bpcol-1 gene should present a high risk of cross-reactivity"*. Therefore she concluded, *"that the use of this collagen for diagnosis should not be pursued"*. Since her work towards the production of an induced protein had failed, her conclusion has to be considered with caution and represents for us a sufficient motivation for reinvestigating that aspect in order to reach a scientifically valid conclusion. Moreover, results obtained in chapter 2 concerning the specificity of the antisera *Bpcol-1* (a-19-1) for *B. pahangi* led to think that the gene *Bpcol-1* should be used for the study on the antigenicity of cuticular collagen.

In that situation, it is nessesary
a) to prepare a recombinant protein by expressing the gene *Bpcol-1*.
b) to immunize Balb/c mice with the produced protein in order to obtain a specific antiserum *Bpcol-1*.
c) to test the new *a-Bpcol-1* antiserum for its specificity. The confirmation of the specificity could open a fundamental research on the protein's epitopes.

A bacterial system was choosen initially to induce the desired proteins. As no result was obtained the induction was done with an yeast expression system.
The yeast expression system is recognized as an ideal eukaryotic microorganism for biological studies (Guthrie, C. and Fink, G.R., 1995). In spite of its complexity and the presence of the greater size of its genome, it shares with bacteria such as *E. coli* most of the technical advantages: relative rapid growth, ease of plating and replica-plating, ease of mutant isolation and availability of a highly versatile DNA transformation system as well as many other well known molecular tools. Moreover since the gene is provided from an eukaryotic organism, the resulting induced protein should be produced efficiently. *Pichia pastoris* system is a very user-friendly and cheap system. The choice of the *Pichia* Expression kit (Invitrogen), was done in accordance with the recommendations of Dr. Zocchi (Institut of Chemistry, University of Neuchâtel, group of Professor Ward). As eukaryote, *Pichia pastoris* possesses many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification. The technical process is easy and might be compared with those of *E. coli* and *Saccharomyces cerevisiae*. It is faster and less expensive than other eukaryotic expression systems such as baculo-virus or mammalian tissue culture and generally gives higher expression levels. As yeast, it shares the advantages of molecular and genetic manipulations with those of *Sacharomyces* and has the added advantage of 10-100-fold higher heterologous protein expression levels. These features make *Pichia* very useful as a protein expression system.

2 Materials and methods

2.1 Molecular biology material

2.1.1 cDNA library of *Brugia pahangi*

The cDNA library of *Brugia pahangi* was prepared in the phage λUniZap XR from ARNm from L3 gathered 3 days after infection. The library was a kind gift of Dr. Hunter, University of Liverpool, U.K.

2.1.2 PQE_{30} + gene encoded for a 28 kDa protein

The gene inserted into the pQE_{30} vector encoded for a protein of 28 kDa. This construction (obtained from Dr. Bovet from the laboratory of plant physiology of Neuchâtel) will be used as an independent positive control for protein induction with bacteria system.

2.1.3 Bacteria strains

- XL1-Blue-recA$^-$ strain: *E. coli* (recA1, lac$^-$, end A1, gyrA96, thi, hsdR17, supE44, relA1, [F', proAB, lac/q, LacZΔM15, Tn10] (tet^r)). This strain was a kind gift of the biochemistry's laboratory of Neuchâtel.
  XL1-Blue *E. coli* strain was used to replicate pGEM®-T-Easy Vector (Promega) and pQE30 (Qiagen) recombinant vector.

- Strain SG13009[pREP4] (Qiagen) derived from E. coli K12 and have the phenotype Nal^S, Str^{S}m Ruf^S, Thi$^-$, Lac$^-$, Ara$^+$, Gal$^+$, Mtl$^-$, F$^-$, RecA$^+$, Uvr$^+$, Lon$^+$. This strain was used for expression.

- Strain BL21

- Strain BL21 (DE3)

- Strain Tuner (DE3) pLac I

2.1.4 Yeast strain

- GS115 his$^-$ Mut$^+$: *Pichia pastoris* strain (Invitrogen). This strain was a kind gift of the Chemistry Institute of the University of Neuchâtel (group of Prof. Ward).

2.1.5 Vectors

2.1.5.1 Cloning vector

pGEM®-T-Easy Vector (Promega). This vector is used with XL1-Blue-recA$^-$ strain.

2.1.5.2 Bacteria expression vector

2.1.5.2.1 The pET vectors

The pET vectors were originally constructed by Studier and colleagues (Studier and Molfatt, 1986; Rosemberg *et al.*, 1987; Studier *et al.*, 1990). The newer pET derivatives were designed with enhanced features to permit easier subcloning, detection and purification of target proteins. Induction was tried with two different pET vectors:

- pET 9a (amp^r) used with BL21 (DE3) strain derived from *E. coli*.
- pET blue-2 (amp^r) used with Tuner (DE3) pLac I derived from *E. coli.*

2.1.5.2.2 The pQE vectors

High-level expression of 6xHis-tagged proteins in *E. coli* using the QIAexpress pQE vectors (Qiagen) is based on the T5 promoter transcription translation system. The pQE plasmid belongs to the pDS family of plasmids (Bujard *et al.* 1987) and is derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS (Stüber *et al.*, 1990). The pQE_{30} vector was used during this research (Fig. 1). Restriction map and sequence for the cloning regions of pQE_{30} vector is presented in (Fig. 2). For expression, the vector is used with *E. coli* SG13009 [$pREP_4$] strain. PQE_{30} vector can be stored in XL1-Blue-recA$^-$. The entire sequence information is available at www.qiagen.com.

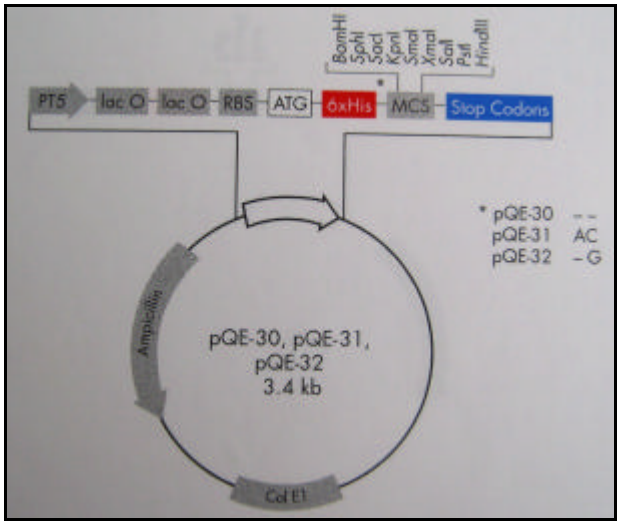

Fig. 1: pQE_{30} vector for N-teminal 6xHis tag constructs. **PT5**: T5 promoter, **lac O**: lac operator, **RBS**: ribosome-binding site, **ATG**: start codons in all three reading frames, **Col E1**: Col E1 origin of replication, **Ampicillin**: ampicillin resistance gene (Qiagen, 2001).

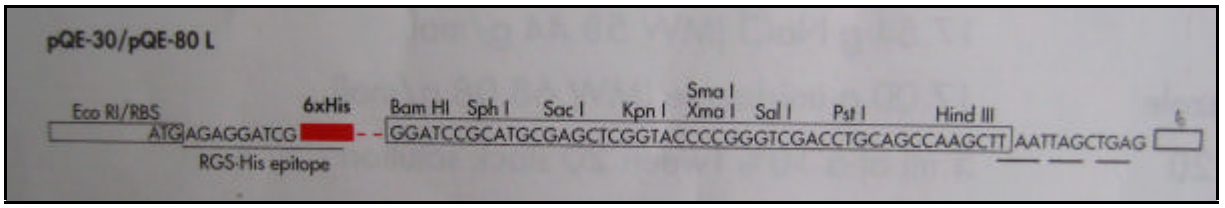

Fig. 2: pQE_{30} vector: multiple cloning sites (Qiagen, 2001).

2.1.5.3 Yeast expression vector

pHIL-S1 expression vector is used in these experiments. This vector is specific for secreted expression. It is a 8260-bp fusion vector with *XhoI, EcoRI, SmaI, BamHI* unique sites.

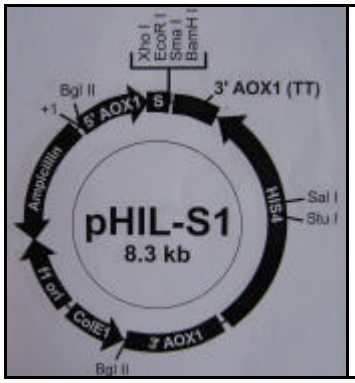

Fig. 3: Map of pHIL-S1. The map shows the location and size of each feature of pHIL-S1.

ColE1: ColE1 origin of replication for bacteria, **f1ori**: f1origin of replication for yeast, **Ampicillin**: ampicillin resistance gene for bacteria, **HIS4**: Histidine resistance gene for yeast, **S**: sequence signal for excreted protein, **AOX1**: Alcohol oxidase promoter.
(Invitrogen, 2000).

2.1.6 Primers

All primers were synthesized by MWG-Biotech (Ebersberg, Deutschland).

Specific primers for vectors

PQE_{30} vector	PQE_{30} 5' primer PQE_{30} 3' primer	5'-ccccgaaaagtgccacctgacgtc- 3' 5'-gttctgaggtcattactggatcta- 3'	T_a = 68°C T_a = 59°C
pGEM®-T-Easy Vector	M13 primer M13 Reverse primer	5'-gtaaaacgacggccagt-3' 5'-ggaaacagctatgaccatg-3'	T_a = 56°C T_a = 56°C

T_a corresponds to the temperature of Annealing

Specific primers for the gene *Bpcol-1*

Bpcol-1 5' primer *Bpcol-1* 3' primer	5'-**ggatcc**atggttgattctgatgatccg- 3' 5'-**cccggg**ttaatagcctggagctgttct- 3'	T_a = 65°C T_a = 68°C

Bold characters correspond to restriction sites.

Bpcol-1 5' and 3' correspond to the flanking region of the collagen gene *Bpcol-1* preceded respectively by the restriction sites *BamHI* and *XmaI*.

Specific primers for the gene *Bpcol-1* used in the yeast expression

Bpcol-1 5' yeast primer	5'-c**ggatcc**catcaccatcaccatcacatggttgattctgatgatccg- 3'	T_a = 70°C
Bpcol-1 3' yeast primer	5'-c**ggatcc**ttaatagcctggagctgttct 3'	T_a = 68°C

Bold characters correspond to *BamHI* restriction sites.

Bpcol-1 5' yeast primer corresponds to the flanking region of the collagen gene *Bpcol-1* preceded first by the 6xHis tag (red coloured) and then from the restriction sites *BamHI*. *Bpcol-1* 3' yeast primer corresponds to the flanking region of the collagen gene *Bpcol-1* preceded by the restriction sites *BamHI*.

2.2 Molecular biology methods

The methods used are PCR, DNA digestion, Purification of DNA fragments, Ligation, Transformation and Sequencing.
All molecular biology methods used in this chapter are the same as those used in chapter 3. Please refer to this chapter for more details.

2.2.1 Selection of the coding sequence of the gene *Bpcol-1* for bacterial expression

Before proceeding to the PCR on the cDNA library, it is necessary to precipitate the cDNA with an aqueous solution of sodium acetate and a solution of EtOH 100%. The amplification of the gene *Bpcol-1* from the cDNA library of *B. pahangi* was done by PCR. For the amplification of the gene *Bpcol-1*, the primers *Bpcol-1* 5' and 3' were used.

cDNA precipitated	5µl
Bpcol-1 5' primer	5µM
Bpcol-1 3' primer	5µM
Nucleotide tri-phosphates	200µM
Reaction buffer	10X
TaqDNA polymerase	1 Unit
Reaction volume	50µl

PCR program

1)	95°C	10min	Initial denaturation	
2)	95°C	30 sec	Denaturation	
3)	63°C	30 sec	Annealing	
4)	72°C	1min	Elongation	Steps 2-4 (35 cycles)
5)	72°C	5min		
6)	10°C			

2.2.2 Selection of the coding sequence of the gene *Bpcol-1* for yeast expression

The selection of the gene *Bpcol-1* is carried out by PCR directly on pGEM® + *Bpcol-1*. To isolate the gene *Bpcol-1*, the primers *Bpcol-1* 5' yeast and *Bpcol-1* 3'yeast were used.

pGEM® + *Bpcol-1* DNA	1µl
Bpcol-1 5' yeast primer	5µM
Bpcol-1 3' yeast primer	5µM
Nucleotide tri-phosphates	200µM
Reaction buffer	10X
TaqDNA polymerase	1 Unit
Reaction volume	50µl

PCR program

1)	95°C	3 min	Initial denaturation	
2)	95°C	30 sec	Denaturation	
3)	52°C	30 sec	Annealing	
4)	72°C	1,5 min	Elongation	Steps 2-4 (10 cycles)
5)	95°C	30 sec	Denaturation	
6)	63°C	30 sec	Annealing	
7)	72°C	1,5 min	Elongation	Steps 5-7 (30 cycles)
8)	72°C	10 min		
9)	10°C			

2.2.3 Bacterial expression system

Process	Detail
Prepare pQE_{30} vector	1. Digestion with restriction enzymes 2. Gel purify
↓	
Prepare insert DNA	3. Plasmid preparation and /or PCR 4. Restriction digest 5. Gel purify
↓	
Clone insert into pQE_{30} vector	6. Ligate insert with pQE_{30} vector 7. Transform into non-expression host (e.g. XL1) 8. Identify positive clones: colony PCR, miniprep, verify reading frame by sequencing.
↓	
Transform into Expression Host	9. Transforming in SG13009 Competent cells

Table provided from Manieri (2002)

Finally, the selected colonies were tested by PCR with appropriate primers:

DNA template (colony)	Bacteria dissolved in water
Bpcol-1 5' primer	3µM
Bpcol-1 3' primer	3µM
Nucleotide tri-phosphates	200µM
Reaction buffer	10X
TaqDNA polymerase	1 Unit
Reaction volume	30µl

PCR program

1)	95°C	1min	Initial denaturation	
2)	95°C	30 sec	Denaturation	
3)	T_a°C	30 sec	Annealing	
4)	72°C	1min	Elongation	Steps 2-4 (30 cycles)
5)	72°C	10min		
6)	10°C			

2.2.3.1 Culture medium

LB	Tryptone	10 g
	Yeast extract	5 g
	NaCl	10 g
Completed to 1 L, autoclave		

2.2.3.2 Cell growth and harvest

The LB medium was inoculated with 1/20 of its volume with an overnight saturated preculture of freshly transformed SG13009 bacteria strain. Ampicillin (100µg/ml) and Kanamycin (25µg/ml) antibiotics were added and bacteria were grown under vigorous shaking until the OD_{600} 0,5-0,7 was reached. Expression of the recombinant protein was then induced with IPTG (Biosynth AG) at a final concentration of 1*mM*. Cells were harvested by centrifugation (3200g at 4°C during 15 min) after the culture had reached the end of exponential growth phase.

2.2.3.3 Analysis of protein: SDS-PAGE and Immunoblotting

To follow the expression by SDS-PAGE and immunoblotting, samples of 1 ml of the culture were withdrawn at regular intervals. The cells were harvested by centrifugation (12000 g during 30 sec) and suspended into loading buffer. The samples were heated for 3 min at 100°C before proceeding to SDS-PAGE (See more details in chapter 2). The protein bands were transferred to nitrocellulose membrane by semidry blotting and immunodetected using cuticular collagen antibodies and anti-His antibodies (Table 1 p. 66). Immunocomplexes were detected using secondary antibodies coupled to peroxidase and visualized by chromogenic reaction with 4-chloro-1-naphtol (See more details in chapter 2).

2.2.4 Yeast expression system

As the yeast expression system is a system that was never used in Betschart's lab, the descriptive methods are presented in the form of protocols in order to ensure the durability of this system in the laboratory.

2.2.4.1 Culture medium

Solid YP glycerol	**Nutrient**	**Amount**
	Peptone	10 g
	Yeast extract	5 g
	Glycerol	1 %
	Agar	10 g
Completed to 500 ml with H_2O, autoclave		

Solid YP methanol	**Nutrient**	**Amount**
	Peptone	10 g
	Yeast extract	5 g
	Agar	10 g
Completed to 450 ml with H_2O, autoclave		
Add sterile methanol 10% just before casting the Petri plates		100 ml

YP glycerol	**Nutrient**	**Amount**
	Peptone	10 g
	Yeast extract	5 g
	Glycerol	1 %
Completed to 500 ml with H_2O, autoclave		

YP methanol	**Nutrient**	**Amount**
	Peptone	10 g
	Yeast extract	5 g
Completed to 450 ml with H_2O, autoclave		
Add sterile methanol 10% just before using this media		100 ml

Amino acide mix powder	**Nutrient**	**Amount in powder (g)**
	Ade	0,63
	URA	0,30
	L-Arg (HCl)	0,30
	L-Asp	1,50
	L-Glu (mono Na^+)	1,50
	L-Leu	0,90
	L-Lys (mono HCl)	0,45
	L-Met	0,30
	L-Phe	0,75
	L-Ser	5,63
	L-Thr	3,00
	L-Trp	0,60
	L-Tyr	0,45
	L-Val	2,25
Use at 1,3 g/l. Store powder at 4°C in a bottle surrounded with blue silica gel.		

Selection maintenance medium plate SC-HIS$^-$	Nutrient	Amount in 500 ml
	Nitrogen base-aa	3,35 g
	Amino acide mix powder	0,65 g
Dissolve in nanopure water. Adjuste the pH to 6 with KOH		
	Bacto agar	10 g
	Nanopure water	Fill to 450 ml
Autoclave and cool. Before dispensing add the following:		
	20 % glucose	50 ml
If necessary, adjust to final volume with sterile nanopure water. Store at –20°C in the dark.		

Induction medium plate SC MeOH-His$^-$	Nutrient	Amount in 500 ml
	Nitrogen base-aa	3,35 g
	Amino acide mix powder	0,65 g
	Nanopure water	Fill to 350 ml
	Agar	10 g
Autoclave and cool. Before dispensing add the following:		
	Sterile MeOH 10%	50 ml
	Sterile K_2HPO_4 1M	6,6 ml
	Sterile KH_2PO_4 1M	43,4 ml
	Sterile nanopure water	Fill to 500 ml
The solution is buffered to pH 6 by the phosphate buffer. Store at –4°C in the dark. Plates are sealed separately with parafilm.		

2.2.4.2 Preparation of competent cells

1. Two days before the experiment, 5 ml of YP (Glu) medium are inoculated with a single yeast colony of the strain to be transformed. The culture is placed overnight to saturation at 30°C.
   The saturated culture may be prepared up to several weeks in advance and stored at 4°C.
2. The night before transformation, a 2-liter flask containing 500 ml of YP (Glu) is inoculated with an appropriate amount (1/5000 of the initial culture volume) of the saturated culture and grown overnight with vigorous shaking at 30°C to an OD_{600} of 0,7 – 1. This OD is used if the electroporation cuvette (Bio Rad) has a 0,1 cm-gap cuvette. If a 0,2 cm-gap cuvette is utilized, the OD must be 1,3 – 1,5.
3. The culture is harvested by centrifuging at 4°C, 1500 x g for 5 min, resuspended in 500 ml of sterile distilled water and centrifuged another time at 4°C, 1500 x g for 5 minutes. If using a culture volume different from 500 ml, resuspend in the same volume.
4. The cells are washed and concentrated 3 times by centrifuging at 1500 x g for 5 minutes, resuspending the successive pellets as follows:
   - First pellet: 250 ml (or ½ of the initial volume) ice cold sterile water,
   - Second pellet: 20 ml ice cold sterile 1*M* sorbitol. Transfer to a 50 ml sterile Falcon tube,
   - Third pellet: 0,5 ml ice cold sterile 1*M* sorbitol. In this case the final volume (cells + sorbitol) must be ~ 750 µl. If initial culture volume is different from 500 ml this pellet must be resuspended in 1:1000 of the initial volume.

Resuspension should be vigorous enough to completely dissociate each pellet. The final volume of resuspended yeast should be ~1 ml and final OD_{600} should be~200. This final

volume is enough for 25-37 transformation reactions. If possible, it is better to avoid the preparation of a frozen glycerol stock of electrocompetent yeast cells, as the transformation efficiency will drop by a factor of 10 with freezing. The volumes of concentration and washing solution can be changed, but it is important to maintain always the ratios among first flask, culture volume, harvesting volume, washing first and third pellets.

5. The concentrated yeast culture is transferred in a sterile, ice-cold 1,5 ml Eppendorf tube.

2.2.4.3 Electro-transformation and plating (Micropulser, Bio Rad)

The *Bpcol-1* gene was expressed in GS115 cells. Therefore GS115 cells were transformed by electro-transformation with pHIL-S1+ *Bpcol-1* expression vector. Before the transformation it is necessary to linearize pHIL-S1+ *Bpcol-1* with *Sal* I restriction enzyme. After the transformation it is needful to incubate the plates at 30°C until colonies appear. These colonies will be used for the induction.

1. The sterile electro-cuvettes (Bio Rad) are removed from their pouches and placed on ice. The white chamber slide is placed on ice.
   Alternatively the whole apparatus is placed in a cold chamber.
   The following 2-6 steps are performed as quickly as possible.
2. In a cold 0,5 ml Eppendorf tube, 40 µl of the cell suspension was mixed with 2,5 – 5 µl of linearized DNA (DNA should be in TE at a concentration of 1-2 µg/µl). Mix well and transfer in an ice-cold electroporation cuvette. Let sit on ice 5 minutes.
3. The *E. coli* pulser apparatus is set to 1,5 KV when using the 0,2 cm-gap cuvettes. It is set to 0,75 KV when using the 0,1 cm-gap cuvettes.
4. The cuvette is placed in a chilled safety chamber slide. The slide is pushed into the chamber until the cuvette is seated between the contacts in the base of the chamber.
5. Pulse once.
6. The cuvette is removed from the chamber and 1 ml of 1*M* sorbitol is immediately added to the cuvette and the cells are quickly, but gently resuspended with a thin Pasteur pipette. (*This rapid addition of sorbitol after pulse is very important in maximizing the recovery of the transformant*).
7. The pulse parameters are checked and recorded. The time constant should be between 4 and 5 milli seconds. The field strength can be calculated as actual Kilovolts (KV)/cuvette gap (cm).
8. 400 µl are plated on selection plates and incubated at 30°C, till colonies appear.

2.2.4.4 Liquid induction

1. Using a single colony, 5 ml of YP (Glu) are inoculated in a 50 ml Falcon tube. The culture is placed overnight at 30°C in a shaking incubator (250 rpm).
2. In a 2 liters flask, 500 ml YP (Glu) are prepared and 500 µl preculture is added. The culture is placed for an additional 24 hours at 30°C.
3. The cells are harvested by centrifuging at 2000 x g for 15 minutes. The supernatant is decanted and the cell pellet is resuspended in 500 ml YP + 1% MeOH to induce expression.

4. The culture is placed in a 2 liters flask and covered with 2 layers of sterile gauze. The culture is placed again into the incubator to continue the growth.
5. MeOH is added to a final concentration of 1% every 12 hours during 60 hours to maintain induction.
6. The supernatant is transferred to a separate tube. The supernatant and the cell pellets are stored at –20°C until ready to dot blot or SDS-PAGE and Immunoblotting.

2.2.4.5 Analysis of protein: SDS-PAGE and Immunoblot.

To follow the expression by SDS-PAGE and immunoblotting, 80 µl of the supernatant was mixed to 20 µl of SDS-PAGE 5x Gel Loading buffer. The samples were heated for 3 min at 100°C before proceeding to SDS-PAGE (See more details in chapter 2). The protein bands were transfered to nitrocellulose membrane by semidry blotting and immunodetected using a-120colA.s antibodies and anti-His antibodies (Table 1 p.66). Immunocomplexes were detected using secondary antibodies coupled to peroxidase and visualized by chromogenic reaction with 4-chloro-1-naphtol (See more details in chapter 2).

2.3 General methods

2.3.1 Immunoblotting with a-His antibodies

The membrane was blocked with a buffer (3% BSA in TBS buffer) for 1 hour. After washing twice for 10 min in TBS-Tween/Triton buffer and for 10 min with TBS buffer, the nitrocellulose membrane was incubated for 1 hour in antibody solution, diluted in the blocking buffer. After washing twice for 10 min each time in TBS-Tween/Triton buffer and for 10 min in TBS buffer, the membrane was incubated with anti-mouse secondary antibody peroxidase conjugate diluted 1/1000 in 3% BSA in TBS for 1 hour. After washing 4 times for 10 min each time in TBS-Tween/Triton buffer, the membrane was stained with peroxidase staining solution until the signal is clearly visible. The chromogenic reaction was stopped by rinsing the membrane twice with water. All these steps were performed at room temperature.

Remarks: Buffer containing milk powder should not be used for Anti-His Antibody that would reduce the sensitivity of the process.

TBS (Tris Buffered Saline):	10 *mM* Tris HCl, pH 7,5 150 *mM* NaCl
TBS-Tween buffer:	20 *mM* Tris-HCl, pH 7,5 500 *mM* NaCl 0,05% (v/v) Tween 20
TBS-Tween/Triton buffer:	20 *mM* Tris-HCl, pH 7,5 500 *mM* NaCl 0,05% (v/v) Tween 20 0,2% (v/v) Triton X-100

Peroxidase staining solution: TBS; 3 mg/ml 4-chloro-1-naphtol (in methanol); 0,2% 30% hydrogen peroxide (H_2O_2)

2.3.2 Production of a-*Bpcol-1* antibodies

The production of antibodies raised against the recombinant *Bpcol-1* cuticular collagen protein was made using Balb/c mice (from the animalhouse of the Institute of Zoology of Neuchâtel). The purification of the recombinant proteins *Bpcol-1* (60 kDa, 66 kDa and 90 kDa proteins) was made by electroelution (see more details in chapter 2). The obtained purified recombinant proteins were used for the immunization. Before the immunization process, a blood sample of 100µl was taken from the mice by a retro orbital puncture as negative control. Balb/c mice were immunized by a subcutaneous injection of 100µg antigen in Freund's incomplete adjuvant followed by another similar subcutaneous infection every 2 weeks. The blood was recovered 2 months after the initial immunization by the bleeding of mice. The blood was then incubated at room temperature during a few hours and then centrifuged for 10 minutes at 3000 rpm. Sera containing the antibodies were recovered and frozen at –20°C.

2.3.3 Antisera

Name	**Primary antibodies**	**Secondary antibodies**
a-120colA.s (produced by myself)	Mouse/anti *A.suum* cuticular collagen 120kDa	Goat/anti-mice (IgG, H+L) peroxidase
Bpcol-1 (a-19-1)	Mouse/anti *Brugia pahangi* cuticular collagen specific gene *Bpcol-1 (19-1)*	Goat/anti-mice (IgG, H+L) peroxidase
B. pahangi (a-205)	Rabbit/anti *Brugia pahangi* cuticular collagen	Goat/anti-rabbit (IgG, H+L) peroxidase
Anti-His	Mouse/anti 6xHis tag	Goat/anti mice (IgG, H+L) peroxidase
a-*Bpcol-1* (produced by myself)	Mouse/anti *Brugia pahangi* recombinant cuticular collagen *Bpcol-1*	Goat/anti-mice (IgG, H+L) peroxidase

Table 1: List of primary and secondary antibodies used during the research.

3 Results

3.1 Initial attempts to produce recombinant Bpcol-1 cuticular collagen proteins

The initial attempts to produce recombinant *Bpcol-1* cuticular collagen proteins were carried out by Di Mito using the pGEX vector (Pharmacia Biotech). As she did not obtain recombinant proteins, the induction was first tried using the pET vector system. Induction with pET system gave no result. The stability of the construct (insert + vector) was weak and bacteria transformed with that construct do not grow. Finally the pQE_{30} vector system was used for the expression in bacteria.

3.2 Selection of the coding sequence of the gene Bpcol-1 for the expression with a bacterial system

The gene *Bpcol-1* was amplified by PCR using directly a cDNA library of *B. pahangi*. The analysis on agarose gel of the PCR results showed a band around 900-bp corresponding to the size of the gene *Bpcol-1* (Martin *et al.*, 1996) (Fig. 4). The purification of the 900-bp fragment, its ligation into the pGEM®-T-Easy vector and its transformation into XL1 Blue cells were performed. A selection of white colonies was verified by PCR with M13 and M13 reverse primers. Agarose gel analysis of the PCR shows bands around 1000-bp that correspond to the size of the gene (900-bp) plus a part of the pGEM®-T-Easy vector (Fig. 5). The nature of the clones was also verified by digestion with *BamHI* and *XmaI* enzymes. The agarose gel analysis of the digested clones shows one band of 900-bp corresponding to the size of the gene *Bpcol-1* and one band of 3000-bp corresponding to the size of the vector (Fig. 6). The single digestion with *BamHI* gives a band of 3900-bp (Fig. 6). The sequence of the isolated gene was also verified to insure that the isolated gene is the gene *Bpcol-1* (Table 2).

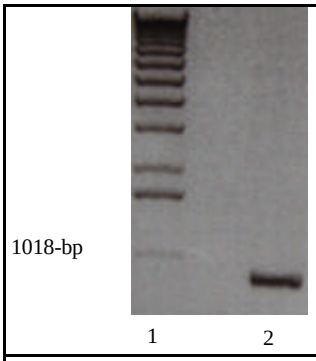

Fig. 4: **PCR**: lane 1: DNA ladder; lane 2: *Bpcol-1* DNA obtained by PCR amplification with *Bpcol-1* 5' and 3' primers.

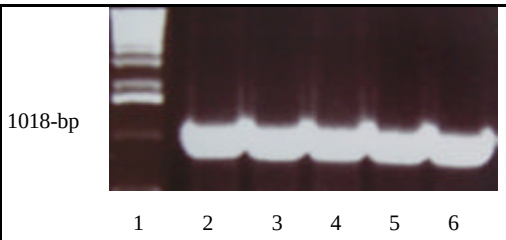

Fig. 5: **PCR**: lane 1: DNA ladder; lanes 2-6: *Bpcol-1* DNA obtained by PCR amplification with M13 and M13 reverse on a selection of white transformed colonies.

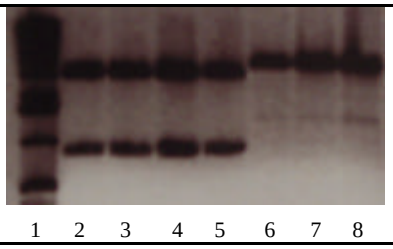

Fig. 6: **Digestion of pGEM® + *Bpcol-1***: lane 1: DNA ladder; lanes 2-5: pGEM® + *Bpcol-1* digested with *BamHI* and *XmaI*; lanes 6-8: pGEM® + *Bpcol-1* digested with *BamHI*.

```
pGem+Bpcol-1     1 CATGGCGGCCGCGGGAATTCGATTGGATCCATGGTTGATTCTGATGATCCGAAACAGCTT
Bpcol-1          1 -GT---A----C-AAG-TT-AG-TAG----ATGGTTGATTCTGATGATCCGAAACAGCTT
consensus        1 --*--------*-----**----*-*----*****************************


pGem+Bpcol-1    61 CTCATTGAGGCAGAAAGCATGAAAAAGCTTGCCTTTTGCGGTGTTGCTGTTTCTACCGTA
Bpcol-1         53 CTCATTGAGGCAGAAAGCATGAAAAAGCTTGCCTTTTGCGGTGTTGCTGTTTCTACCGTA
consensus       61 ************************************************************

pGem+Bpcol-1   121 GCTACTCTGGTAGCAATTATTTGCGTACCAATGCTCTGCACCTACATGCAAAATGTGCAG
Bpcol-1        113 GCTACTCTGGTAGCAATTATTTGCGTACCAATGCTCTGCACCTACATGCAAAATGTGCAG
consensus      121 ************************************************************

pGem+Bpcol-1   181 TCTAACTTGCAAGATGAGATTAGCTTCTGCAGGACCCGCGCAATTGGATTACGAGGAGAA
Bpcol-1        173 TCTAACTTGCAAGATGAGATTAGCTTCTGCAGGACCCGCGCAATTGGATTACGAGGAGAA
consensus      181 ************************************************************

pGem+Bpcol-1   241 TTCACCAAACTCGAATCATCGCGTTCAGCACTGAAAAAAGAAAGACAAAAGAGGCAGGCA
Bpcol-1        233 TTCACCAAACTCGAATCATCGCGTTCAGCACTGAAAAAAGAAAGACAAAAGAGGCAGGCA
consensus      241 ************************************************************

pGem+Bpcol-1   301 GTATTCCAATGTTGCAGTTGCGGTATTGGTCCTGTTGGTCCACCAGGCCCACCTGGACAA
Bpcol-1        293 GTATTCCAATGTTGCAGTTGCGGTATTGGTCCTGTTGGTCCACCAGGCCCACCTGGACAA
consensus      301 ************************************************************

pGem+Bpcol-1   361 GATGGTGACGATGGCCGAGATGGCCCACCTGGAAAACCTGGTATGCCAGGTCAGGATGCT
Bpcol-1        353 GATGGTGACGATGGCCGAGATGGCCCACCTGGAAAACCTGGTATGCCAGGTCAGGATGCT
consensus      361 ************************************************************

pGem+Bpcol-1   421 CAAGAAACCCAACTGCCAACTGAACGAGACTGGTGTTTCAATTGTCCTGCAGGACCACCA
Bpcol-1        413 CAAGAAACCCAACTGCCAACTGAACGAGACTGGTGTTTCAATTGTCCTGCAGGACCACCA
consensus      421 ************************************************************

pGem+Bpcol-1   481 GGTCCACGAGGTAAACCAGGACCAAAAGGACAGAGAGGATTGCCGGGAGACAAAGGTTCT
Bpcol-1        473 GGTCCACGAGGTAAACCAGGACCAAAAGGACAGAGAGGATTGCCGGGAGACAAAGGTTCT
consensus      481 ************************************************************

pGem+Bpcol-1   541 AGCGGACAGCCTGGTGAACCGGGTCCTGTGGGACCACAAGGGCCAAGAGGACCGAATGGA
Bpcol-1        533 AGCGGACAGCCTGGTGAACCGGGTCCTGTGGGACCACAAGGGCCAAGAGGACCGAATGGA
consensus      541 ************************************************************

pGem+Bpcol-1   601 CCTCGAGGAAATCCAGGCCCAGCTGGCGAACCAGGAAAACCGGGTGTACAGACTGAGGTG
Bpcol-1        593 CCTCGAGGAAATCCAGGCCCAGCTGGCGAACCAGGAAAACCGGGTGTACAGACTGAGGTG
consensus      601 ************************************************************
```

```
pGem+Bpcol-1  661 CCTGGACCACCAGGACCACCTGGCCCACCAGGACCACAAGGACCACCAGGTGAGCAAGGT
Bpcol-1       653 CCTGGACCACCAGGACCACCTGGCCCACCAGGACCACAAGGACCACCAGGTGAGCAAGGT
consensus     661 ************************************************************

pGem+Bpcol-1  721 CCAGCTGGGCGAGATGGCAATCCCGGACGACCAGGACCACGTGGACCACCAGGTCAGAAC
Bpcol-1       713 CCAGCTGGGCGAGATGGCAATCCCGGACGACCAGGACCACGTGGACCACCAGGTCAGAAC
consensus     721 ************************************************************

pGem+Bpcol-1  781 GGCAAAGATGGTCCTCCAGGACATGATGGTCCAAATGGTGATCAAGGAGAAGCAGGTCCC
Bpcol-1       773 GGCAAAGATGGTCCTCCAGGACATGATGGTCCAAATGGTGATCAAGGAGAAGCAGGTCCC
consensus     781 ************************************************************

pGem+Bpcol-1  841 GATGGACCAAAAGGCAGTTGTGACCATTGTCCACCTCCCAGAACAGCTCCAGGCTATTAA
Bpcol-1       833 GATGGACCAAAAGGCAGTTGTGACCATTGTCCACCTCCCAGAACAGCTCCAGGCTATTAA
consensus     841 ************************************************************

pGem+Bpcol-1  901 CCC-------GGGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGA
Bpcol-1       893 -C--------GGGA-T--C-AG-AGA-T-A-----ACCTG--A-TCA--C---TAG-A--
consensus     901 -*---------****-*--*-**---*-*--------****----**---*---*-*-*--

pGem+Bpcol-1  954 GCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCA
Bpcol-1       946 --TC-----G--T----T-CAT--C----AT--TC-----TAT-A
consensus     961 --**-----*--*----*-***--*-----*--**-----*-*-*
```

Table 2: Sequence alignment of *Bpcol-1* gene (Martin, S.A. *et al*., 1996) with the gene isolated in 3.2 from *B. pahangi* cDNA library and inserted into pGEM® vector. Restriction sites *BamHI* and *XmaI* are boxed in gray color. Start and stop codons are bolded.

3.3 Verification of the construction of the pQE_{30} expression vector with Bpcol-1 insert

30-50 ng of pGEM®-T-Easy + *Bpcol-1* vectors were digested with 1 U of *BamHI* & *XmaI* and after analysed on agarose gel (Fig. 7). One 900-bp fragment, corresponding to the gene *Bpcol-1*, was ligated into *BamHI* & *XmaI* digested pQE_{30} expression vector (Fig. 7). After the transformation into XL1 Blue cells, a selection of colonies was checked by PCR with pQE_{30} 5' and 3' primers in order to verify the nature of the clone. Agarose gel analysis of the PCR shows bands around 1200-bp corresponding to the size of the gene *Bpcol-1* + a part of the pQE_{30} vector (Fig. 8). The nature of the clones was also verified by a digestion with *BamHI* and *XmaI* enzymes. The digestion analysis by agarose gel shows one band of 900-bp corresponding to the size of the gene *Bpcol-1* and one band of 3400-bp corresponding to the size of the vector. Single digestion gives a band around 4300-bp (Fig. 9). The sequence of the clone was done to check the reading frame (Table 3).

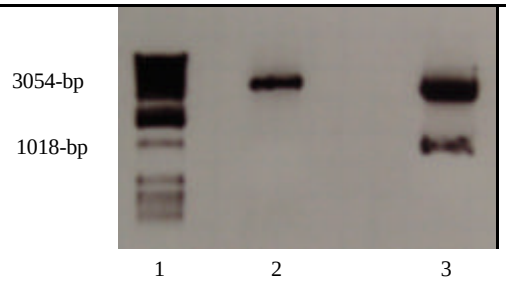

Fig. 7: **Digestion**: lane 1: DNA ladder; lane 2: pQE_{30} vector digested with *BamHI* and *XmaI.* lane 3: pGEM® + *Bpcol-1* digested with *BamHI* and *XmaI*.

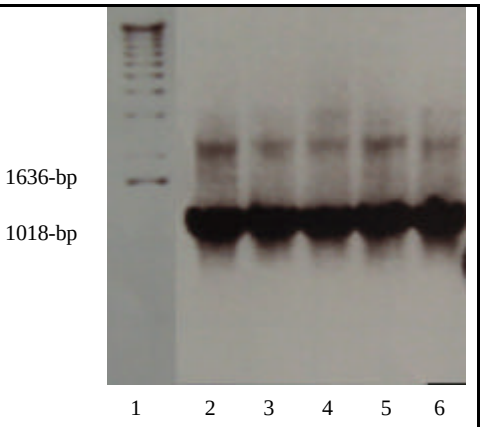

Fig. 8: **PCR on XL1-Blue transformed colonies**: lane 1: DNA ladder; lanes 2 to 6: 900-bp *Bpcol-1* + 336-bp of pQE_{30}. DNA obtained by PCR amplification with pQE_{30} 5' and 3' primers.

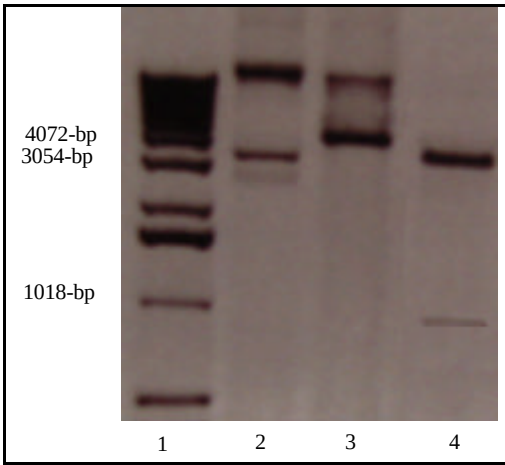

Fig. 9: **Digestion**: lane 1: DNA ladder; lane 2: pQE_{30} + *Bpcol-1* undigested; lane 3: pQE_{30} + *Bpcol-1* digested with *BamHI*; lane 4: pQE_{30} + *Bpcol-1* digested with *BamHI* and *XmaI*.

```
   1 attgtgagcg gataacaatt tcacacagaa ttcattaaag aggagaaatt aactatgaga
  61 ggatcgcatc accatcacca tcacggatcc atggttgatt ctgatgatcc gaaacagctt
 121 ctcattgagg cagaaagcat gaaaaagctt gccttttgcg gtgttgctgt ttctaccgta
 181 gctactctgg tagcaattat ttgcgtacca atgctctgca cctacatgca aaatgtgcag
 241 tctaacttgc aagatgagat tagcttctgc aggacccgcg caattggatt acgaggagaa
 301 ttcaccaaac tcgaatcatc gcgttcagca ctgaaaaaag aaagacaaaa gaggcaggca
 361 gtattccaat gttgcagttg cggtattggt cctgttggtc caccaggccc acctggacaa
 421 gatggtgacg atggccgaga tggcccacct ggaaaacctg gtatgccagg tcaggatgct
 481 caagaaaccc aactgccaac tgaacgagac tggtgtttca attgtcctgc aggaccacca
 541 ggtccacgag gtaaaccagg accaaaagga cagagaggat tgccgggaga caaaggttct
 601 agcggacagc ctggtgaacc gggtcctgtg ggaccacaag ggccaagagg accgaatgga
 661 cctcgaggaa atccaggccc agctggcgaa ccaggaaaac cgggtgtaca gactgaggtg
 721 cctggaccac caggaccacc tggcccacca ggaccacaag gaccaccagg tgagcaaggt
 781 ccagctgggc gagatggcaa tcccggacga ccaggaccac gtggaccacc aggtcagaac
 841 ggcaaagatg gtcctccagg acatgatggt ccaaatggtg atcaaggaga agcaggtccc
 901 gatggaccaa aaggcagttg tgaccattgt ccacctccca gaacagctcc aggctattaa
 961 cccgggtcga cctgcagcca agctt
```

Table 3: Sequence map of pQE_{30} + *Bpcol-1* clone. Restriction sites *BamHI* and *XmaI* are boxed in red color and gray coloured nucleotides indicate the 6xHisTag region. Start and stop codons are bolded.

3.4 Expression

In order to express the *Bpcol-1* gene in SG13009 [$pREP_4$] cells, the cells were transformed with pQE_{30} + *Bpcol-1* expression vector. A selection of colonies was tested by PCR with *Bpcol-1* 5' and 3'. Small cultures of positive clone (PCR amplified fragment of 900-bp) were grown in LB liquid culture containing Ampicillin and Kanamycin and induced with 1*mM* IPTG. The same preparation was done with pQE30 + 28 kDa expression vector as the positive control.

The curve obtained in Fig. 10A corresponds to the usual growth curve of the bacteria SG13009 [$pREP_4$] strain. Bacteria transformed with the expression vector pQE30 without any insert have the same growth curve that those observed when the bacteria are not transformed (Fig 10B). The growth curve of bacteria culture transformed with pQE30 + 28 kDa expression vector presents small differences but corresponds to usual growth curve (Fig. 10C). However the growth curve of bacteria transformed with pQE_{30} + *Bpcol-1* is unusual (Fig. 10D). The growth is slowed down.

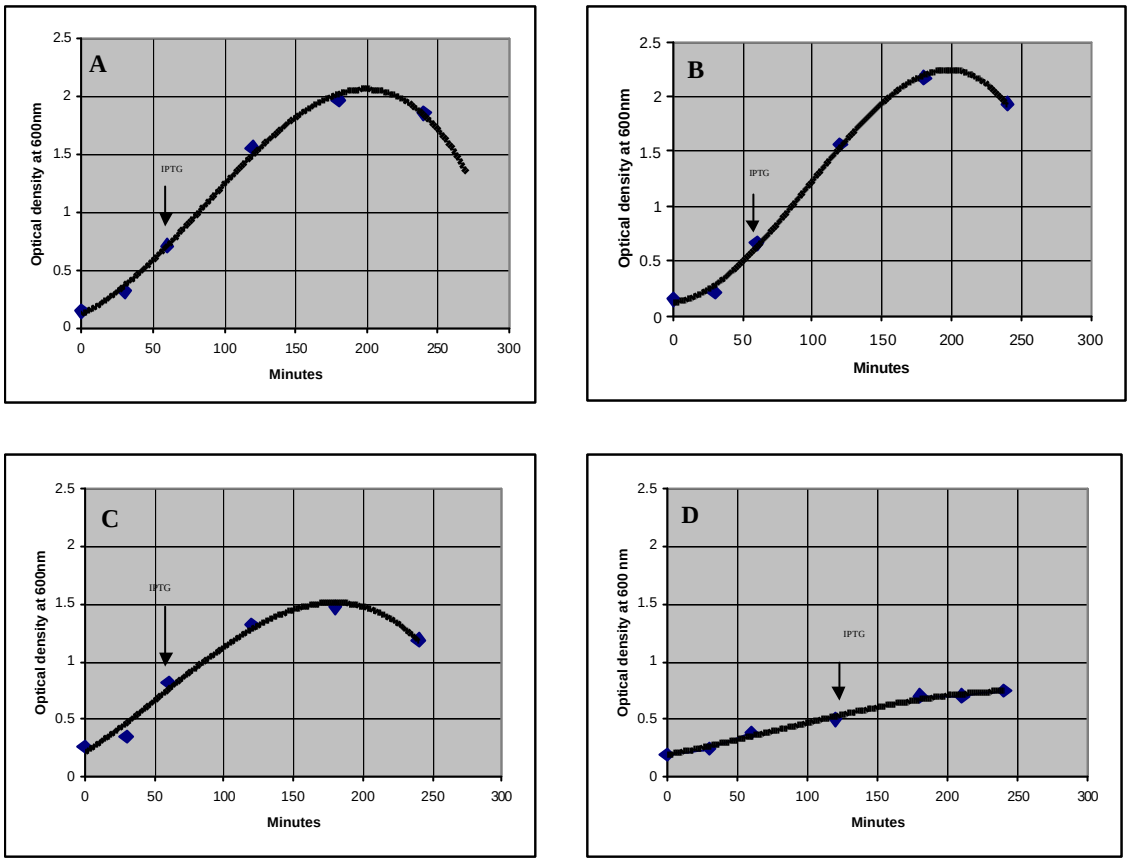

Fig. 10: **A.** Bacteria growth of SG13009 [pREP$_4$]. **B.** Bacteria growth of SG13009 [pREP$_4$] transformed with pQE$_{30}$ before and after IPTG. **C.** Bacteria growth of SG13009 [pREP$_4$] transformed with pQE$_{30}$ + 28 kDa before and after IPTG. **D.** Bacteria growth of SG13009 [pREP$_4$] transformed with pQE$_{30}$ + *Bpcol-1* before and after IPTG. Cell density was followed by measuring the absorbance at 600 nm and IPTG was added after 60 min of growth (120 for **D.**) (arrow). Culture was stopped at the end of the exponential growth phase.

3.5 The characterization of the protein Bpcol-1

3.5.1 Electrophoretic analysis

The protein profile analysis of the induced culture SG13009 [pREP$_4$] transformed with pQE$_{30}$ + *Bpcol-1* does not show any induction. The protein profiles of the induced transformed bacteria show the same protein profiles as those observed with the bacteria transformed with the vector only (Fig. 11). The protein profile of induced bacteria transformed with pQE30 + 28 kDa reveals a band around 30 kDa. This band corresponds to the recombinant protein of 28 kDa (Fig. 12).

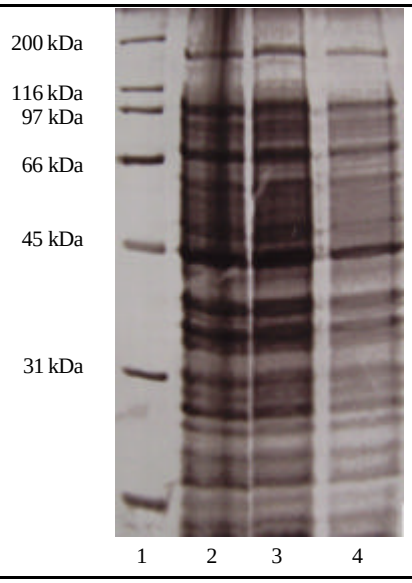

Fig. 11: **SDS-PAGE analysis of culture**. Lane 1: HMW; lane 2: SG13009[pREP$_4$], transformed with pQE$_{30}$ without insert, before IPTG; lane 3: SG13009[pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1*, before IPTG; lane 4: After 180 min IPTG;

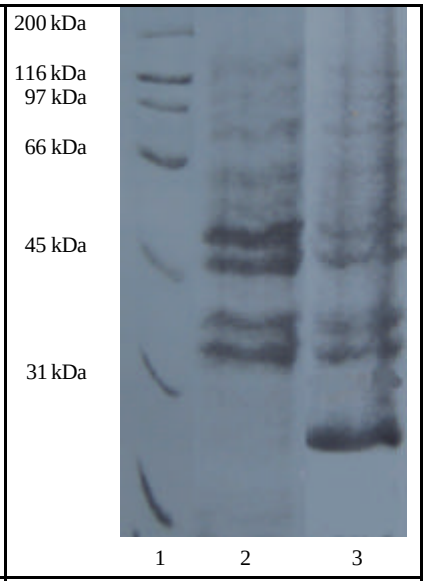

Fig. 12: **SDS-PAGE analysis of culture**. Lane 1: HMW; lane 2: pQE$_{30}$ + 28 kDa before IPTG; lane 3: pQE$_{30}$ + 28 kDa after 180 min IPTG.

3.5.2 Immunoblotting analysis

3.5.2.1 Detection of proteins with anti cuticular collagen antibodies

The antisera a-120colA.s, *Bpcol-1* (a-19-1) and *B. pahangi* (a-205) were used to test the induction of the protein *Bpcol-1*.

When tested with a-120colA.s antibodies, immunoblot results on induced bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1*, reveal 3 bands, one around 34 kDa and two others around 60 and 90 kDa. When tested with *B.pahangi* (a-205) antibodies, two bands appear around 60 and 90 kDa. No bands were detected when tested with *Bpcol-1* (a-19-1) antibodies (Fig. 13).

Immunoblot with antibodies a-120colA.s, on induced bacteria extract, digested with collagenase or pepsine, reveal the same bands as those observed before the digestion (Fig. 13).

Immunoblot on non-induced bacteria strain, transformed with pQE$_{30}$ + *Bpcol-1*, shows the same results as those obtained after induction (Fig. 15).

Results obtained in Fig. 14 reveal that immunoblot with a-120colA.s on induced bacteria extract SG13009 [pREP$_4$] transformed with pQE$_{30}$ vector reveals 3 bands apparently of identical molecular size as those observed on bacteria extract SG13009 [pREP$_4$] transformed with pQE$_{30}$ + *Bpcol-1* vector. Immunoblot with a-120colA.s on a culture growth of bacteria extract SG13009 [pREP$_4$] shows also the same 3 bands.

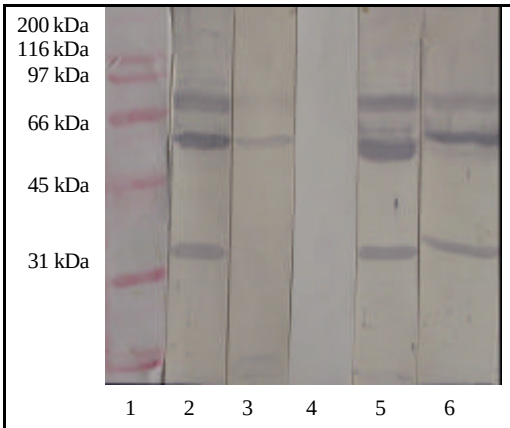

Fig.13: Western blot analysis on bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1*, after 180 min IPTG: lane 1: HMW; lane 2: WB with antiserum a-120colA.s; lane 3: WB with antiserum *B. pahangi* (a-205); lane 4: WB with antiserum *Bpcol-1* (a-19-1); lanes 5 and 6: WB with antiserum a-120colA.s on bacteria extract digested respectively with collagenase and pepsine.

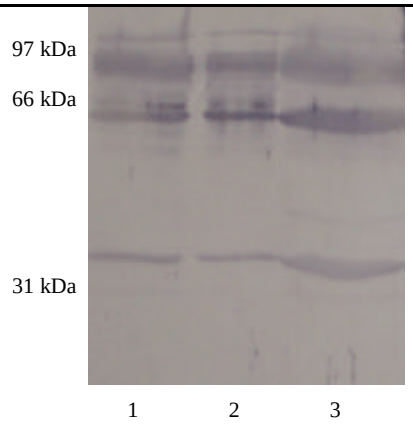

Fig.14: Western blot analysis with antiserum a-120colA.s: lane 1: WB on bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1*, after 180 min IPTG; lane 2: WB on bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30,}$ after 180 min IPTG; lane 3: WB on bacteria extract SG13009 [pREP$_4$] after a culture growth of 4 hours.

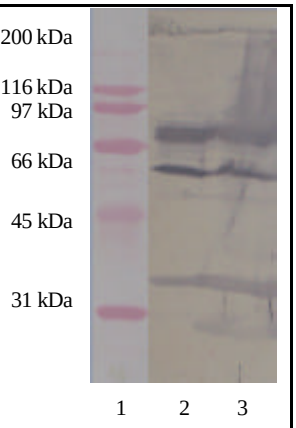

Fig.15: Western blot analysis with antiserum a-120colA.s on bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1*: lane 1: HMW; lane 2: After 180 min IPTG; lane 3: Before induction.

3.5.2.2 Detection of proteins with anti-His antibodies

Immunoblot with anti His antibodies on induced bacteria extracts SG13009 [pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1* vector shows no reaction (Fig. 16). However, immunoblot on induced bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + 28 kDa vector, revealed a band of 30 kDa (Fig. 16). Control immunoblots on induced bacteria not transformed or on induced bacteria transformed with the vector pQE30 only show no reaction with anti-His antibodies. No reaction was found on induced and non-induced bacteria transformed with pQE$_{30}$ + *Bpcol-1* vector (Fig. 17).

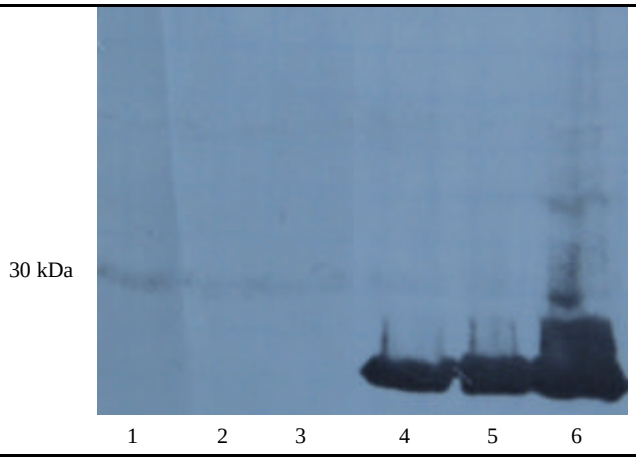

Fig.16: **Western blot analysis** with Anti-His antibodies: lanes 1-2-3: Bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1*, after 60,120,180 min IPTG; lanes 4-5-6: Bacteria extract SG13009 [pREP$_4$] transformed with pQE$_{30}$ + 28 kDa, after 60, 120, 180 min IPTG.

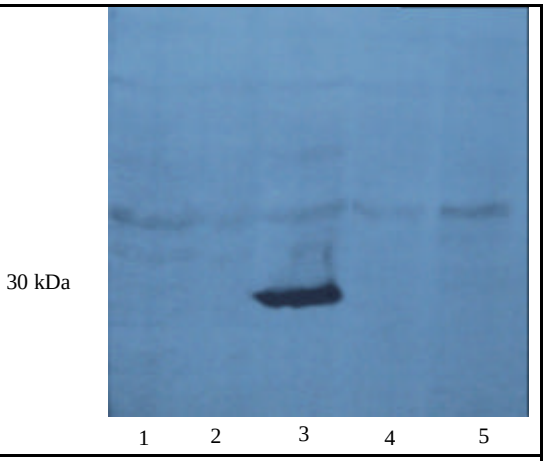

Fig.17: **Western blot analysis** with Anti-His antibodies: lane 1: Bacteria extract SG13009 [pREP$_4$]; lane 2: Bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$, after 180 min IPTG; lane 3: Bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + 28 kDa, after 180 min IPTG. lane 4: Bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1*, before IPTG; lane 5: Bacteria extract SG13009 [pREP$_4$], transformed with pQE$_{30}$ + *Bpcol-1*, after 180 min IPTG

3.6 Selection of the coding sequence of the gene Bpcol-1 for the expression with the yeast system

The gene *Bpcol-1* was amplified by PCR using directly a purified pGEM® + *Bpcol-1* vector. The analysis on agarose gel of the PCR result shows a band around 900-bp corresponding to the size of the gene *Bpcol-1* (Fig. 18). The purification of the 900-bp fragment, its ligation into the pGEM®-T-Easy vector and its transformation into XL1 Blue cells were performed. A selection of white colonies was checked by PCR with *Bpcol-1* 5' yeast and *Bpcol-1* 3' yeast primers. Agarose gel analysis of the PCR shows bands around 900-bp (Fig. 19). The nature of the clones was also verified by digestion with *BamHI* enzyme. The agarose gel analysis of the digested clones shows one band of 900-bp corresponding to the size of the gene *Bpcol-1* and one band of 3000-bp corresponding to the size of the vector (Fig. 20).
The sequence of the isolated *Bpcol-1* gene was also verified. Results are the same as those obtained in Table 2, paragraphe 3.2 exept for the presence of the 6xHis tag before the ATG start codon. The presence of the 6x His tag is important for the future detection of the recombinant protein.

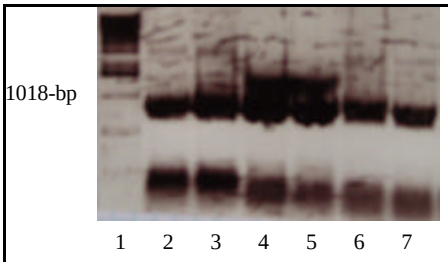

Fig. 18: **PCR**: lane 1: DNA ladder; lanes 2 to 7: *Bpcol-1* gene obtained by PCR amplification with *Bpcol-1* 5' yeast and 3' yeast primers.

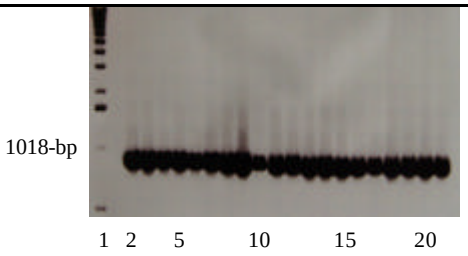

Fig. 19: **PCR**: lane 1: DNA ladder; lanes 2 to 21: *Bpcol-1* DNA obtained by PCR amplification with *Bpcol-1* 5' yeast and 3' yeast primers on a selection of white transformed colonies.

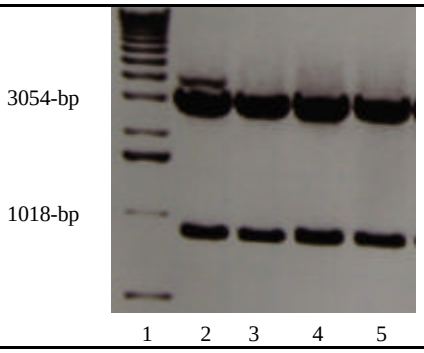

Fig. 20: **Digestion of pGEM® + *Bpcol-1***: lane 1: DNA ladder; lanes 2 to 5: pGEM® + *Bpcol-1* digested with *BamHI*.

3.7 Verification of the construction of the pHIL-S1 expression vector with Bpcol-1 insert

30-50 ng of pGEM®-T-Easy + *Bpcol-1* vectors were digested with 1 U of *BamHI* and analysed on agarose gel (Fig. 20). The 900-bp fragment corresponding to the gene *Bpcol-1* was ligated into *BamHI* digested pHIL-S1 expression vector (Fig. 21). After the transformation into XL1 Blue cells, a selection of colonies was checked by PCR with *Bpcol-1* 5' yeast and *Bpcol-1* 3' yeast primers in order to verify the nature of the clone. Agarose gel analysis of the PCR shows bands around 900-bp (Fig. 22). The nature of the clones was also verified by a digestion with *EcoRI* enzyme to check the correct insertion way. The correct clones have 2 bands, one of 9000-bp and one of 200-bp (Fig. 23).

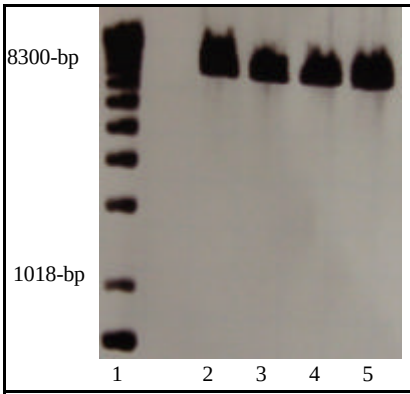

Fig. 21: **Expression vector after digestion**: lane 1: DNA ladder; lanes 2 to 5: pHIL-S1 vector digested with *BamHI*.

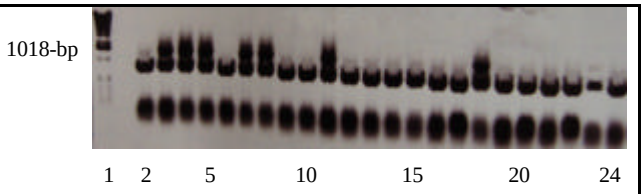

Fig. 22: **PCR on XL1-Blue transformed colonies**: lane 1: DNA ladder; lanes 2 to 24: 900-bp *Bpcol-1* DNA obtained by PCR amplification with *Bpcol-1* 5' yeast and *Bpcol-1* 3' yeast primers.

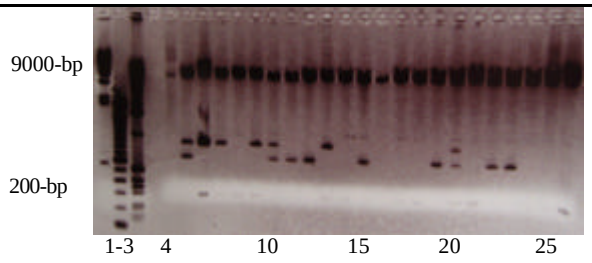

Fig. 23: **Digestion**: lanes 1-2-3: Different DNA ladders; lanes 4 to 26: pHLI-S1 + *Bpcol-1* digested with *EcoRI*.

3.8 The characterization of the protein Bpcol-1

3.8.1 Electrophoretic analysis

The protein profile of the induced yeast culture (supernatant part) transformed with pHIL-S1 + *Bpcol-1* shows a lot of bands ranging from 20 kDa to 200 kDa. 3 stronger bands appear between 50-90 kDa (Fig. 24). The same induced yeast culture digested with collagenase enzyme reveals a different protein profile. The 3 bands, observed before the digestion, are digested and new bands appear especially around 35 kDa and 50 kDa. Bands around 116 kDa correspond to collagenase (Fig. 24). A negative control corresponding to induced yeast culture (supernatant part) transformed only with pHIL S1 vector is represented on Fig. 24.

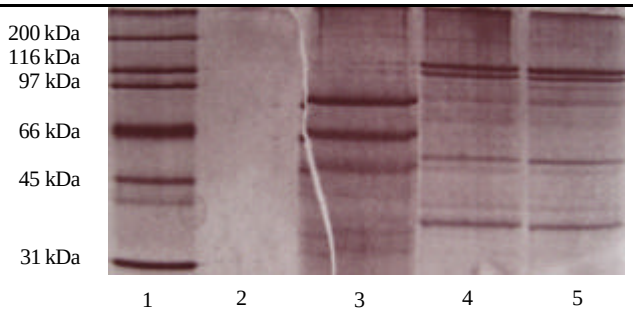

Fig. 24: **SDS-PAGE analysis**: Lane 1: HMW; lane 2: Negative control lane (induced yeast culture without insert); lane 3: Induced yeast culture; lanes 4 and 5: Induced yeast culture digested with collagenase.

3.8.2 Immunoblotting analysis

3.8.2.1 Detection of proteins and immunoblotting analysis with anti *Bpcol-1* sera

a-120colA.s antibody and Anti 6x His tag antibody were tested on induced and non-induced yeast culture (supernatant part) in order to verify the production of the recombinant protein *Bpcol-1*. Immunoblotting on the supernatant of induced yeast culture with anti His antibodies reveals only two bands instead of three but confirms the induction. The a-120colA.s antibody recognized mainly the higher band and only slightly the lower band (Fig. 25).
Immunoblot with *B. pahangi* (a-205) antibodies on the recombinant protein *Bpcol-1* reveals 2 bands around 60 and 90 kDa. These bands have the same molecular weight as those observed on immunoblot with a-120colA.s and Anti 6x His tag antibodies (Fig. 26).
Antibodies produced against the *Bpcol-1* protein produced in *Pichia* were used for immunoblot on the recombinant protein *Bpcol-1*, on *A. suum* and on *C. elegans* cuticular collagen (Fig. 27). Results obtained on the recombinant protein show 2 bands around 60 and 90 kDa. These bands have the same molecular weight as those observed on SDS with other antibodies (Fig. 25 and 26). No band is visible for immunoblot on *A. suum* and *C. elegans* cuticular collagen.
No band is visible for immunoblot on the recombinant protein digested with collagenase and tested with a-*Bpcol-1* antibody (Fig. 28).

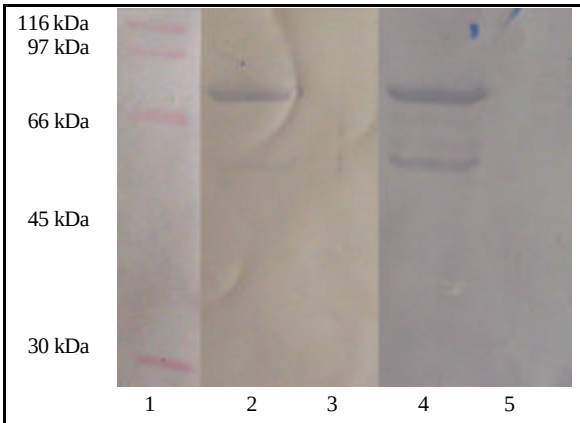

Fig. 25: Western blot analysis on induced and non-induced yeast culture (supernatant part): lane 1: HMW; lane 2: WB with antibodies a-120colA.s on induced culture; lane 4: WB with Anti His antibodies on induced culture; lanes 3 and 5: WB with antibodies a-120colA.s and Anti-His antibodies on non-induced culture.

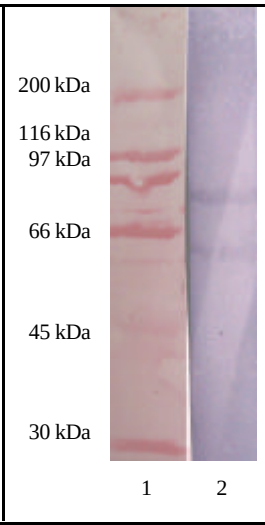

Fig. 26: Western blot analysis with a-205 *B. pahangi* antibodies. Lane 1: HMW; lane 2: WB on induced culture.

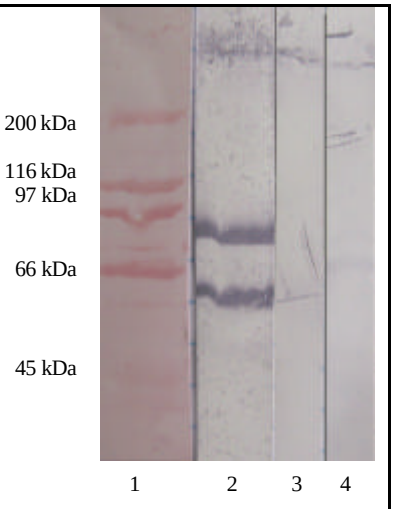

Fig. 27: Western blot analysis with the produced antibodies a-*Bpcol-1*. Lane 1: HMW; lane 2: WB on induced culture; lane 3: WB on *A. suum* cuticular collagen; lane 4: WB on *C. elegans* cuticular collagen.

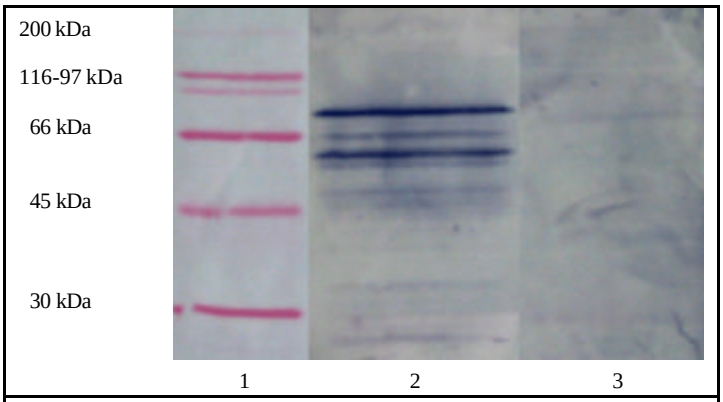

Fig. 28: Western blot analysis with the produced antibodies a-*Bpcol-1*. Lane 1: HMW; lane 2: WB on induced culture; lane 3: WB on induced culture digested with collagenase.

4 Discussion

During the expression of the entire *Bpcol-1* gene in a bacterial system, it was difficult to reach an OD_{600} of higher than 0,5. However the growth of the bacteria cultures, transformed with pQE_{30} + the insert equivalent to a 28 kDa protein, transformed with pQE_{30} only as well as non transformed cells, reached in the same time periode higher OD levels. Despite these difficulties, SDS-PAGE and immunoblot using the induced cultures were performed. The positive control (pQE_{30} + the insert equivalent to a 28 kDa protein) could be induced. As expected, no induction could be found in the bacterial culture transformed with pQE_{30} + *Bpcol-1*. However, it was interesting to observe reactions on the immunoblot when tested with different antisera raised against cuticular collagens. The bands around 34 kDa, 60 and 90 kDa indicate the possibility of a positive induction of collagen proteins.
Immunoblot reactions on non-induced bacteria transformed with pQE_{30} + *Bpcol-1* as well as on induced bacteria transformed with pQE_{30} + *Bpcol-1* were identical when tested with a-120colA.s antibody. Collagenase and pepsin digestion of the induced bacteria extracts did not change the antibody reactions. This leads to think that the proteins recognized, are not collagen. Moreover, immunoblot reaction with a-120colA.s on induced bacteria transformed with pQE_{30} only, as on non-transformed bacteria reveal the same bands. These results clearly indicate the non-specificity of the antibodies and therefore the need to use another antibody to confirm the presence of an induction.
The anti 6x His antibody can distinguish induced clones with 6x His tagged protein from those that only express the short peptide sequence lacking an insert. Small peptides (< 30 amino acids) expressed from pQE_{30} vectors without an insert are degraded within the cells and will not give a positive signal. The lack of the reaction with the induced bacteria extract confirms that the induction was not successful and that the positive antibody reactions initially found were an artefact. However, with the positive control (pQE_{30} + the insert equivalent to a 28 kDa protein) a strong reaction occured at 28 kDa confirming the induction of the recombinant 28 kDa protein. It seems evident that the presence of *Bpcol-1* gene affects the host cell's growth rate and those results seem to lead to the conclusion that *Bpcol-1* gene might be toxic.
By using yeast as a second expression system, the gene *Bpcol-1* was inserted into the expression vector pHIL-S1 using *BamHI* cloning site and including the 6x His-tag sequence. This allows detection of induced proteins using anti His antibodies.
Induction of the yeast transformed with the *Bpcol-1* gene results in culture supernatants with three major bands between 45-97 kDa, whereas supernatants of induced cultures without insert show no protein bands. The digestion with collagenase of the proteins present in the supernatant of the induced culture resulted in the degradation of these three proteins. This clearly indicates that the recombinant proteins are collagens.
Immunoblotting on these proteins present in the supernatant of induced yeast culture with anti His antibody reveals instead of three, only two bands. The a-120colA.s antibody recognized mainly the higher band. The a-205 *B. pahangi* antibody directed against cuticular collagens also reacted with two bands and finally the *a-Bpcol-1* antibody raised against the recombinant collagen proteins reacted strongly with two bands. The specificity of this antibody was shown since no reaction on *A. suum* or *C. elegans* cuticular collagens could be detected.

Taken together, the data of the immunoblot analysis indicate that only two bands of the three are recognized. In contradiction to these results is the fact that in SDS PAGE all three bands disappear after collagenase digestion.
The immunoblot using *a-Bpcol-1* antibodies on the proteins present in the supernatant without or with digestion confirms that induced peptides are collagenase sensitive. In addition, the antibody reacted not only with the two major bands but reveals at least three additional weak bands all of them also collagenase sensitive.
This data seem to indicate that the induced proteins cannot be in a reproducible way separated on SDS PAGE.
The *Bpcol-1* inserts codes for a 30 kDa protein. However on the gel no such peptide can be found. The size of the induced peptides varies in the range of 40 to 90 kDa. If a polymerisation occurs spontaneously in the yeast system, a protein band arround 60 kDa has to be found. In fact the strong bands around 66 kDa could correspond to a dimer. The band below 97 kDa could potentially be a trimer. No clear-cut explanation can be given for the other bands. It is known that the yeast culture system favors polymerisation of induced proteins (*Pichia* Expression kit Invitrogen), a fact, which could explain the absence of the 30 kDa band. How can the presence of multiple collagenase sensitive peptides be explained? One hypothesis might be that the proteins are posttranslationnaly modified, for instance via the cleavage of signal peptides or by specific glycosylation.
It is clear on the basis of our data that dimer and trimer forms of polypeptide chains can be issued from proteins of the same gene. *In vivo* it is not known whether polypeptide chains issued from different genes can polymerize to each other in order to form the triple helical chain of the collagen proteins.
The sucessful production of specific antibodies (a-*Bpcol-1)* raised against the recombinant protein *Bpcol-1* confirms for the first time the immunogenicity of the collagen proteins issued from one gene, however it is not clear whether the antibodies recognize monomer. It might well be that the antigens used were spontaneously polymerized.
Further investigations are necessary to better understand the mechanism of protein synthesis and assembly. Such studies would eventually allow opening new ways for a control of nematode parasites (Fetterer and Rhoads, 1993; Pritchard *et al*., 1988). Our study could be complemented by tests using the antibodies a-*Bpcol-1* on *B. pahangi* cuticular collagens, to extend the production of recombinant collagens to *A. suum* and *C. elegans* and to cross check the produced antibodies for their specificities. For this type of study new cDNA of *A. suum* and *B. pahangi* would be needed to test the presence of identical genes.

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1 General discussion

Nematodes are organisms with a cylindrical body that is not segmented; they are also known as round worms. They can be free-living organisms or they can be found as endoparasites. When they are parasites, they can cause important damage to their host, especially to the human being, as it is the case for lymphatic filariidae, for example
Wuchereria bancrofti is an example of filariidae that invades the lymphatic vessels of their host. The disease caused by this parasite, which is known as lymphatic filariasis, causes the dysfunctioning of the lymphatic system and can, after considerable time of infection, lead to a deforming and mutilating disease of the limbs and genitals. Some 120 million people are currently estimated to be infected: one third of them live in India, one third in Africa and most of the remainder in South Asia, in the Pacific and in the Americas. Lymphatic filariasis cans thus be considered as one of the most important diseases in the world. The extent of this phenomenon has led the WHO (World Health Organization) to create a program, which aims at controlling the transmission and the development of this disease. This program now appears to have brought lymphatic filariasis under control. Still, as the diagnosis techniques are not optimal, as no vaccine is available as yet, and as the presently available treatments (DEC, Ivermectin, Albendazol) remain harmful to the human, as, considerable research work still remains to be devoted to lymphatic filariidae. Moreover, since parasites are adaptable organisms, they may obviously become resistant to current treatments (WHO, 1997).
The successful establishment of a parasite clearly depends on the nature of the interactions between the parasite and its host; (Betschart, 1990; Bird and Bird, 1991) we therefore decided to work on the cuticle, which is the location of these interactions. As more than 80 % of the cuticle is made of collagen proteins, particular attention was given to cuticular collagens. It was also observed that antibodies against cuticular collagens can be found in hosts infected by filariidae (Selkirk *et al.*, 1989; Selkirk and Blaxter, 1990), so that is also appeared necessary to study the immunological aspect of collagens.

After developing a comparative study of protein profiles of cuticular collagens in three different nematodes (*A. suum*, *C. elegans*, *B. pahangi*), we proceeded to study the immunogenicity and the antigenicity of the cuticular collagens, which led us to the production

of recombinant proteins. After antibodies had been raised against these proteins, the possibility of isolating identical proteins in *A. suum* and *B. pahangi* was finally investigated.

1. Comparative Study of Protein Profiles of Cuticular Collagens in Three Different Nematodes (*A. suum, C. elegans, B. pahangi*)

The protein profiles of cuticular collagen in *A. suum, C. elegans* and *B. pahangi* analyzed by SDS-PAGE reveal molecular sizes from 30 kDa to 200 kDa. Since we are dealing with different species, the proteins also exhibit different profiles, as far as the number and the size of their bands are concerned, our observations confirm the results obtained by Betschart (1990) in his study of cuticular collagen of *A. suum*. Cuticular collagens can be divided into 4 groups according to their size:

- 30 kDa proteins (monomer form)
- 60-80 kDa proteins (dimer form)
- 90-120 kDa proteins (trimer from)
- >200 kDa proteins

These four different groups of proteins are made possible by the formation of polypeptide associated with cystein, tyrosine and lysine cross-links (Cox, 1990). The nature of these proteins was confirmed by collagenase and pepsine digestion. Collagenase and pepsine are two enzymes, which respectively allow the triple helical domain of the collagen to be digested, and the amino acids Phe, Trp, or Tyr to be cut in globular domains. SDS-PAGE analysis of the proteins before and after the digestions reveals different protein profiles, and thus confirms the cuticular collagen nature of the three different nematodes' cuticle extracted proteins.

2. Study of the Immunogenicity of Cuticular Collagens

Immunogenicity and antigenicity studies on cuticular collagen were carried out by injecting cuticular *A. suum* collagen of into Balb/c mice. The recovered mice sera were tested by immunoblot on different nematodes' cuticular collagens. Results show that *A. suum* cuticular collagens are immunogen and that they induce the production of antibodies when injected into mice. Immunoblot on cuticular collagen digested with collagenase moreover revealed that the produced antibodies actually react against cuticular collagen proteins. It was also observed that these antibodies are not specific to their immunogens: cross-reactions occurred when antibodies produced against cuticular collagen of different nematodes species were tested by immunoblot on the cuticular collagen of three nematodes (*A. suum*, *C. elegans*, *B. pahangi*). Except for one affinity antibody reacting against a recombinant cuticular collagen protein of *B. pahangi*, no specificity could be related the antisera.

3. Production of Recombinant Cuticular Collagen Proteins

The production of specific antibodies against recombinant cuticular proteins appeared to be the next objective of our research work. Starting from the *Bpcol-1* gene, which is present in *B. pahangi* and in its encoded cuticular collagens, we inductively tried to produce *Bpcol-1* recombinant proteins, which were then used to immunize Balb/c mice. In order to produce antibodies raised against these recombinant proteins. The resulting antibodies were tested on the cuticular collagen of different nematode species, so that their specificity could be tested.

The bacteria induction system, which we first resorted to soon, revealed a drawback: since bacteria cytoplasm’s is not favorable to disulfide bonds, this system does not favour the formation of cystein cross-links. Still, the induction of the protein in a monomer form could be interesting in the perspective of studying the protein's nature.

The absence of glycosilation in the bacteria can also be a disadvantage for the production of antibodies. As sugars are immunogen, they can favor an immunologic answer after the injection of proteins into mice. Although we know the system could have an influence on the proteins' conformation, we maintained our initial choice of the bacterial induction system and

we realized that it did not yield any satisfactory result. Still, this system allowed us to observe that the *Bpcol-1* gene is toxic for the bacteria: the growing curve reveals that the growth of the bacteria transformed with the vector and the gene *Bpcol-1* is unsatisfactory.

It was finally decided to opt for an induction system from *Pichia pastoris* yeast. This system presents a number of advantages: on the one hand, it appears that *Pichia Pastoris* is a eucaryotic organism, which corresponds to the eucaryotic origin of the *Bpcol-1* gene; on the other hand, the yeast system allows the formation of disulfide bonds, which leads to the production of proteins whose conformation approaches reality; since the proteins' conformation plays an important role in immunogenicity (Invitrogen), this characteristic would constitute an advantage for the production of antibodies raised against the recombinant proteins.

Using a yeast system for the expression of the proteins (Invitrogen) also allows us to produce proteins that will be excreted in the culture media, so that the toxicity problems encountered with the bacterial system can be avoided. Finally, the *Pichia* yeast is the seat of glycosilation. Since collagen proteins are glycosilated proteins, a system inducing glycosilation should yield interesting results. Glycosilation in *Pichia* yeast lead to the addition of 8-14 mannoses, which is not very important, compared to the glycosilation occurring in other yeast systems like *Saccharomyces*, which lead to the addition of 50-150 mannoses (Cereghino *et al.*, 2002). Glycosilation in the collagen protein should be studied in a further experiment. It is important to note that the addition of 8-14 mannoses will bring about less change in the conformation of the produced protein than the addition of 50-150 mannoses.

The proteins obtained after induction in the yeast system have molecular weights comprised between 30-200 kDa when separated on SDS-PAGE. Three groups of proteins stand out as being more important: they reveal proteins of 60-66-90 kDa. The nature of these recombinant proteins was tested by collagenase digestion and the proteins profiles obtained after separation on SDS-PAGE confirm that these recombinant proteins are collagens.

4. Production of Antibodies Raised Against Recombinant Collagen Proteins

The injection of the purified recombinant proteins of the three 60-66-90 kDa groups into Balb/c mice led to the production of antibodies. The recovered sera were tested by immunoblot on the recombinant proteins and on cuticular collagens of *A. suum* and *C. elegans*. The results show that the recombinant proteins are immunogen and that the produced antibodies react against the recombinant proteins. It also appears that these antibodies are specific to their immunogens, as they do not cross-react with cuticular collagens of *A. suum* and *C. elegans.* In order to confirm that the produced antibodies are antibodies raised against cuticular collagens proteins, we tested them by immunoblot on recombinant proteins digested with collagenase. Immunoblot comparison before and after the digestion reveals that the antibodies react against collagens.

5. Search for Identical Proteins in *A. suum* and *B. pahangi*

Finally, we worked with the genes *Ascol-C* and *Bpcol*-1 in order to try and isolate identical proteins among *A. suum* and *B. pahangi*. By PCR, we tried to isolate the gene *Ascol-C* in *B. pahangi* species and the gene *Bpcol-1* in *A. suum* species. The results do not allow us to affirm that identical proteins are present in these two different species. Supplementary researches should be carried out in order to obtain more satisfactory results. New DNA libraries are necessary to exclude the contamination problem. Degenerated primers should also be used in order to isolate similar genes with sequences encoding identical amino acids, but with a different 3rd base.

Our research work on cuticular collagen contributes to releasing knowledge concerning the cuticle, which is the location of the interactions between the parasite and its host. This study, which confirms the immunogenicity of cuticular collagens, has led for the first time to the production of complete recombinant cuticular collagens and of specific antibodies against these proteins.

Considerable work remains to be done on the cuticular collagens of nematodes: the successful production of specific antibodies, in particular, encourages further research in this domain. The creation of the “Filarial Genome Project, set up by different laboratories in the USA, in Great-Britain, India, Indonesia and Egypt, should lead to more cuticular collagen research. In the scope of this project, researchers are investigating a vaccine against *B. malayi*, a lymphatic filariidae; they are also working at improvements of the treatment and at the development of screening tests. Since 1995, this group has isolated 3000 specific new genes to *B. malayi*, and they have also prepared more than 10 DNA libraries. The maintenance of the development cycle of this parasite in laboratory has also been performed in this group.

The search for genes encoding cuticular collagen, among the 3000 *B. malayi* specific genes, as well as the production of recombinant proteins and specific antibodies could constitute possible further research objectives for Professor Betschart’s laboratory.

2 References

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