UNIVERSITÀ DI PARMA

DOTTORATO DI RICERCA IN
SCIENZE MEDICO-VETERINARIE

CICLO XXX

Evaluation of ABCB gene expression in an *Ixodes ricinus* cell line exposed to acaricides

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Anni 2014/2017

To my family

And many nights endure
Without a moon or star
So we will endure
When one is gone and far
(L. Cohen)

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Abstract

Over-expression of ATP-binding cassette (ABC) transporter proteins has been implicated in resistance of ticks to acaricides. Tick cell lines are useful for investigating resistance mechanisms, and development of an *in vitro* model for the study of acaricide resistance would greatly facilitate screening for drug resistance in ticks. In the present study, cultures of the *Ixodes ricinus*-derived cell line IRE/CTVM19 were treated with the acaricides ivermectin, amitraz, permethrin or fipronil to determine cytopathic effects of treatment and modulation of ABC transporter gene expression. In experiment 1 IRE/CTVM19 cells were treated with different concentrations of ivermectin (0, 11, 22 or 33 μM) and incubated for 10 days. Evaluation of viability and relative expression of *ABCB1*, *ABCB6*, *ABCB8* and *ABCB10* genes were carried out at day 10 post treatment. Cell viability ranged between 84% and 92% with no significant differences between untreated and treated cells. Analysis of qRT-PCR results showed that ABC pump expression was not significantly modulated by ivermectin treatment. Expression of the *ABCB8* gene revealed a biphasic trend, based on the ivermectin concentration. *ABCB6* and *ABCB10* gene expression was not modulated by ivermectin treatment and *ABCB1* expression was not detected. In experiment 2, cells were treated with different drug concentrations of ivermectin, amitraz, permethrin or fipronil and incubated for 10 days. Cytology, trypan blue exclusion, MTT assay, and relative expression of ABC (*ABCB1*, *ABCB6*, *ABCB8* and *ABCB10*) genes were determined at day 10 post treatment. Cell morphology was altered following treatment with all drugs, but only at high concentrations. Cell viability, as determined by trypan blue exclusion, was not significantly different from untreated controls ($p>0.1$) following treatment with amitraz and permethrin, but high concentrations of fipronil and ivermectin caused a significant decrease (63% for fipronil and 58% for ivermectin, $p<0.01$). Fipronil and permethrin induced significant ($p<0.01$), dose-dependent reduction on MTT assay at all drug concentrations. Quantitative RT-PCRs showed that the drugs significantly affected expression of ABC genes. Fipronil treatment led to down-regulation of *ABCB1* ($p<0.001$) and up-regulation of *ABCB6*, *ABCB8* and *ABCB10* ($p<0.01$). Amitraz treatment resulted in down-regulation of *ABCB1* (significant difference between 25 μM and 150 μM, $p<0.001$) and up-regulation of *ABCB10* at lower concentrations (50 μM, $p<0.05$). Permethrin led to up-regulation of *ABCB6*, *ABCB8* and *ABCB10* only at 150 μM ($p<0.01$). Ivermectin treatment led to a down-regulation of all the genes under investigation, above all at higher concentrations. The up- and

down-regulation of the expression of different ABC transporter genes detected in IRE/CTVM19 cells, following treatment with amitraz, fipronil, permethrin, and ivermectin supports the proposed application of tick cell lines as *in vitro* models for the study of resistance to these acaricides in ticks.

Introduction

Ticks are obligate blood-sucking arthropods with a global distribution that are able to infect every class of terrestrial vertebrates, including mammals, birds, reptiles, and amphibians. The interest in understanding tick biology and tick-host interactions are due to the increasing importance in human and veterinary medicine. Tick bites are responsible for severe toxic reactions, allergic responses and potentially fatal paralytic tick paralysis. Severe tick infestations in livestock and companion animals may result in serious blood loss or in open wounds in the skin with the risk of secondary infections, or in reduced weight gain, lost milk production, and/or abortion [1-5].

However, the major importance of ticks relies in their potential vector role. Ticks are the second most important vectors of human and animal disease, after mosquitoes, and the first in the number of pathogens they can transmit, with a global economic impact that can be estimated in billions of dollars annually [6]. Tick-borne encephalitis, Lyme disease, Rocky Mountain spotted fever, Mediterranean spotted fever, human granulocytic and monocytic anaplasmosis, and tularemia in humans, as well as babesiosis, theileriosis, anaplasmosis, and ehrlichiosis in livestock are only a brief list of possible tick-borne diseases (TBDs) they can transmit [1-5].

Furthermore, climatic changes detected in the last decades are playing a fundamental role in the change in tick distribution. Numerous studies reported the presence of ticks in northern areas, as well as in higher altitudes [6-15].

Ixodes ricinus is one of the most widespread tick in Europe. The interest in this species is due to several factors. The first is due to its life cycle, because it is a three-host tick, with larvae and nymphs feeding on small and medium mammals, while adults on larger ones, particularly ungulates. Ticks belonging to this species has a wide range of hosts, and the presence of feeding larvae, nymphs, or adults has been reported in more than 300 vertebrate species. *I. ricinus* is the vector of numerous pathogens, including viruses, bacteria, and protozoa [1-5].

The increasing concern for the control of this tick species is due to its spreading in new geographic areas in the north and to areas of higher altitudes. Indeed, *I. ricinus* is the species with the greatest ability of adapting in new environments. Alfredsson *et al*. in October 2017 reported an explicit example of tick diffusion, detecting the presence of questing *I. ricinus* adults and nymphs in southern and southeastern Iceland [14].

Furthermore, changes in human activities are also contributing to tick spread to urban and peri-urban areas. Agricultural policies, for example, are permitting the approach of wild animals (e.g. foxes, wild boars and deer) to city parks and green areas and, along with the increasing interest in outdoor activities, more humans are exposed to a higher risk of tick bites [13]. Indeed, tick and tick-borne diseases are continuing to threaten human and animal health throughout the world.

Historically, numerous studies have been aimed at understanding tick biology in order to control their diffusion and thus the spread of TBDs. Numerous drugs have been developed, and introduced onto the market to solve this issue. However, the continued use of chemicals in veterinary medicine, as well as in agriculture, is leading to the development of drug-resistance, as widely reported for carbamates and organophospates [16-20]. Resistance to other, and relatively recent, molecules have been reported, such as formamidines, pyrethroids, and macrocyclic lactones [21-26].

The mechanisms at the base of drug resistance are many, diverse and often genetically based. They include the absence or modification of drug targets, selection of species-specific structure of targets, induction of several pathways for drugs inactivation or degradation, increased detoxification capacity, as well as decreased drug delivery, and alteration of cell-cycle duration. However, one of the most important mechanism in drug resistance is the expression of efflux pumps [27, 28].

The best-known class of these carriers are the ATP-binding cassette (ABC) transporters, which have been widely studied in resistance to chemotherapeutics in humans. They are highly conserved among kingdoms and their role in resistance mechanisms to several drugs has been reported in ticks of the genus *Rhipicephalus*, while scant information is available on *I. ricinus* [29-31].

Understanding the drug resistance mechanisms in ticks is fundamental for further progress in their control. However, *in vivo* studies are both ethically questionable and economically often not feasible. Indeed, the development and validation of *in vitro* models are necessary. To date, numerous tick cell lines are available from different species and genera. They are mostly derived from embryonic stages of ticks [32]. Tick cell lines were used firstly for cultivation and propagation of tick- and mosquito-borne pathogens, but their importance in understanding of drug-resistance mechanisms is increasing, as reported in studies on *Rhipicephalus* cell lines [33, 34]. No studies on *Ixodes* cell lines have been carried out.

Therefore, in the present PhD project the *Ixodes ricinus* cell line IRE/CTVM19 has been investigated following treatments with several molecules used in tick control (i.e. ivermectin,

amitraz, fipronil and permethrin), in order to analyse the modulation patterns of genes involved in detoxification processes in cells. Particular attention was focused on *ABCB1*, *ABCB6*, *ABCB8*, and *ABCB10* genes, with the aim of characterizing a potential *in vitro* model for the study of drug resistance mechanisms in *Ixodes ricinus* ticks.

1. Ticks

Classification

Ticks belong to the Order Ixodida, Superorder Parasitoformes (Phylum Arthropoda, Class Arachnida, Subclass Acarina - which contain also mites) and are temporary obligated blood sucking arthopods.

At present, 907 species have been identified and grouped into three families [35]:

- Nuttallielidae, which contains only a single species, *Nuttalliella namaqua*;
- Argasidae, also known as “soft ticks”, which includes 186 species;
- Ixodidae, also known as “hard ticks”, the most abundant with 720 recognised species.

The first phylogenetic tree describing the evolution of ticks was published in 1982, even if evolutionary relationships of ticks were speculated since the end of the ‘40s. The increasing use of molecular biology has been important in the understanding of the phylogeny and evolution of ticks during the last twenty years, in particular the Ixodidae, the most abundant family. This growing knowledge has led to several changes in tick nomenclature, and more will likely come in the future. For example, according to molecular phylogenetic studies, the Hyalomminae subfamily should be eliminated, and a new subfamily, Bothriocrotoninae came into existence in 2002 to discriminate an early-diverging lineage of Australian ticks (until now classified in the genus *Aponema*) [35, 36].

Hard ticks are divided into two main morphological and phylogenetic groups: the Prostriata and the Metastriata [3]. The Prostriata are represented by the single subfamily Ixodinae, which consists of the only genus *Ixodes*. In contrast, the Metastriata consists of five subfamilies: Amblyomminae, Bothriocrotoninae, Haemaphysalinae, Hyalomminae and Rhipicephalinae [36, 37]. Of the thirteen described genera in the Metastriata, seven comprise species that are implicated in disease transmission [38]. Moreover, more than 200 species have been reported to feed on people, but relatively few commonly feed on them [39, 40].

Morphology

Ticks have a body structure similar to the Arachnids, as they belong to the same class. Usually, their length varies between 2 to 30 mm (e.g. for some species of the genera *Amblyomma*, *Hyalomma* and *Ornithodoros*), depending on the family and species and the stage of their life cycle. Generally, the body shape of ticks ranges from oval to almost circular. It is flattened in unfed ticks and becomes more similar to an egg (or a drop) in the engorged ones, but differences may be present in males and females, depending on the family they belong to. The presence of important taxonomical elements of the external structure of the tick body is essential for identification.

The tick body is composed of the prosoma (cephalotorax), shared with all arachnids and evident only during the embryonic stages, and the opisthosoma (abdomen), the most distal region, which contains the anal aperture and other specialised structures, such as spiracles (i.e. important for respiratory activity and gas exchanges).

The prosoma region is further subdivided into the capitulum (or gnathosoma, the most proximal region, which correspond to the mouthpart) and the podosoma, which is extended from the end of capitulum to the last leg coxae, thus including legs and genital pore. The podosoma and opisthosoma constitute the so-called idiosoma [1, 3].

The capitulum

The capitulum is a heavily sclerotized structure formed by the hypostome, a paired of 2-segmented chelicerae that merge proximally into the basis capituli and placed among a pair of 4-segmented palps. A non-visible “membrane” separates the hypostome from the chelicerae. The irregular growth of the dorsal and ventral cuticular surfaces of the body affects the capitulum position: it occupies an apical position in unfed ticks and males, while it occupies an anteroventral position in engorged females. It is placed in the same plane of the rest of the body and is joined with the idiosoma through a soft articulation membrane that allows small movements, in particular flexures and extensions.

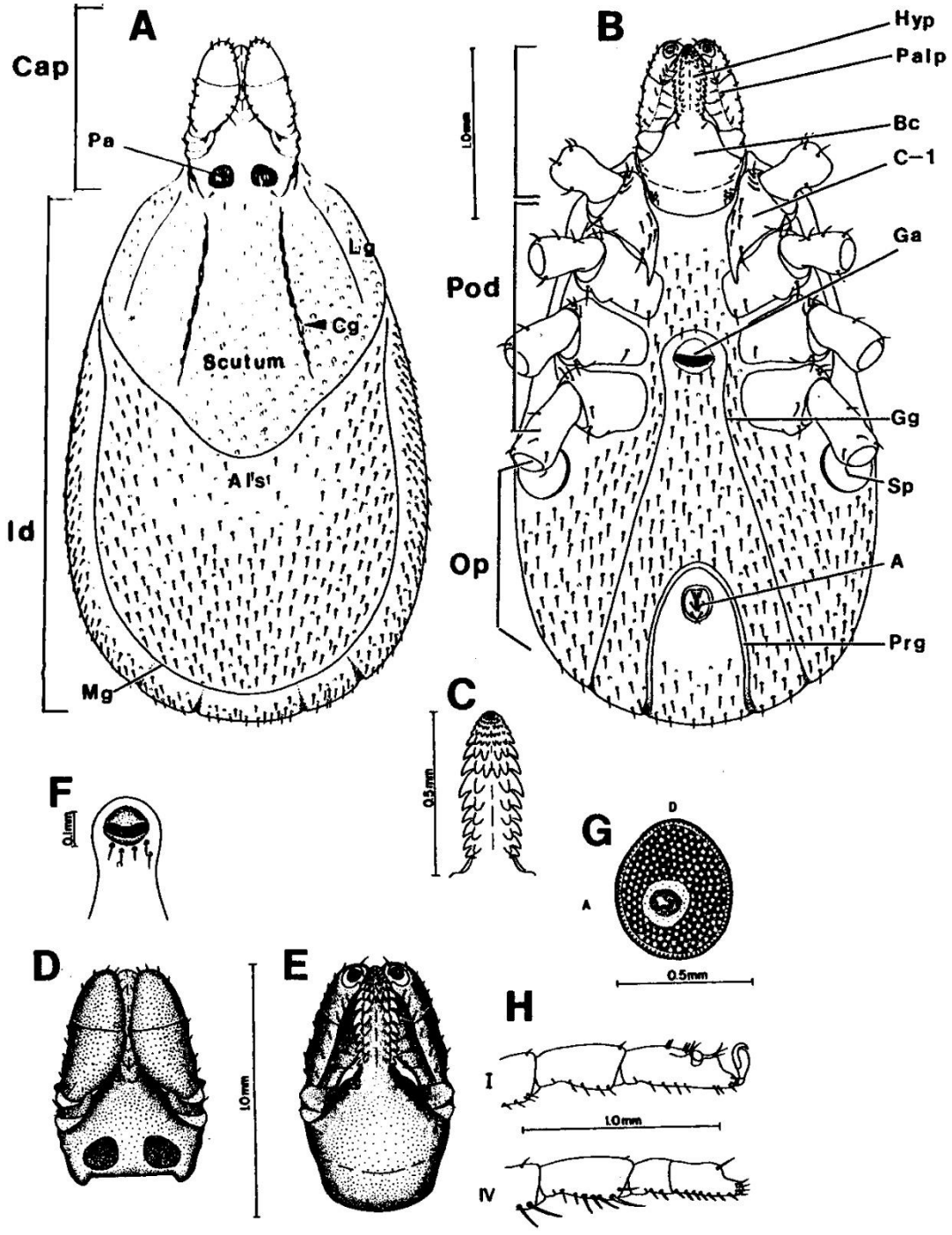


*Figure 1.Diagrams illustrating the external anatomy of a representative ixodid tick female (*Ixodes cookei*). **A** and **B**, dorsal and ventral aspect of tick. **C**, hypostome, ventral view. **D** and **E**, capitulum, dorsal and ventral views. **F**, genital aperture and genital groove. **G**, spiracle. **H**, Terminal segment of leg I. Abbreviations: A (anus); Als (alloscutum); Bc (basis capituli); C-1 (coxa I); CAP (capitulum); Cg (cervical groove); Ga (genital aperture); Gg (genital groove); Hyp (hypostome); Id (idiosoma); Lg (lateral groove); Mg (marginal groove); OP (opisthosoma); Pa (porose area); Pod (podosoma); Prg (preanal groove); Sp (spiracle). Adapted from Sonenshine (1991) [3].*

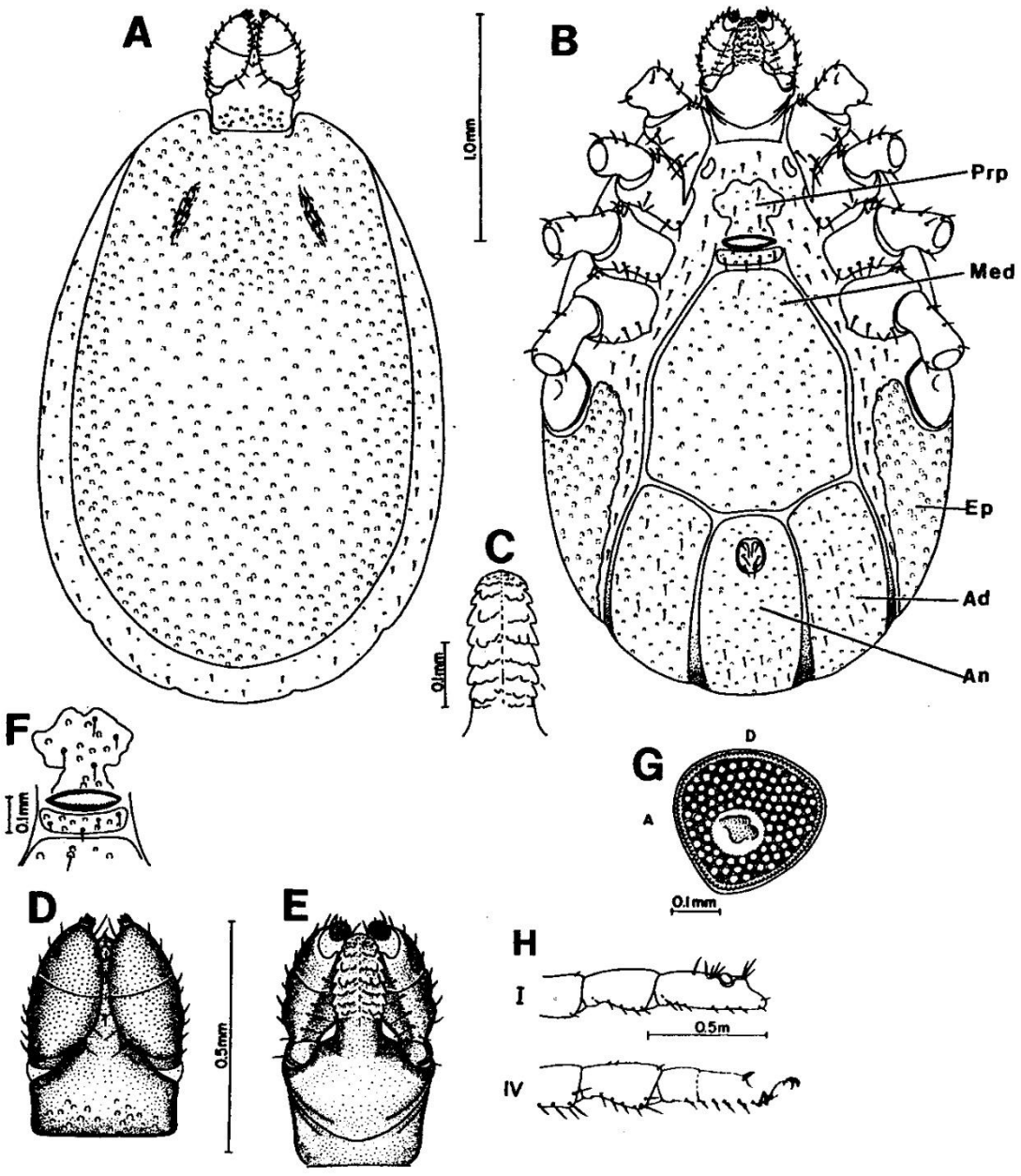


*Figure 2. Diagrams illustrating the external anatomy of a representative ixodid tick male (*Ixodes cookei*). **A** and **B**, dorsal and ventral aspect of tick. **C**, hypostome, ventral view. **D** and **E**, capitulum, dorsal and ventral views. **F**, genital pore, pregenital plate, and adjacent structures. **G**, spiracle. Abbreviations: An (anal plate); Ad (adanal plate); Ep (epimeral plate); Med (median plate; Prp (pregenital plate). Adapted from Sonenshine (1991) [3].*

The hypostome is generally prominent and has a deep groove through which blood drawn from the host passes to the mouth and the pharynx; this groove is known as the 'food canal' (or 'preoral canal') and is located on the dorsal surface of the hypostome. Usually, the hypostome has numerous, curved denticles, but in males of some *Ixodes* species (above all, in nest inhabiting ticks), they can be replaced with faint crenulations.

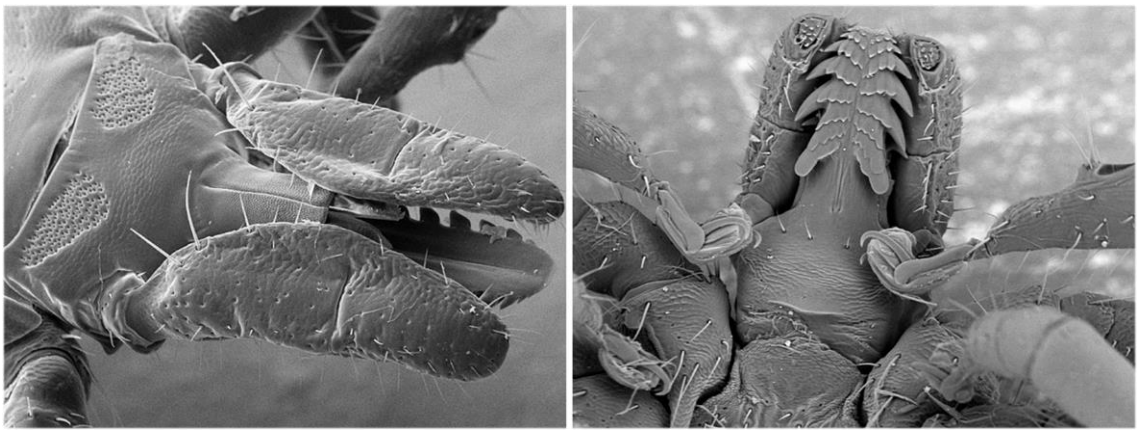


Figure 3. Scanning electron microscopy of Ixodes ricinus *gnathosoma* of female (left, dorsal view) and male (right, ventral view) tick. Female structures are bigger compared to those of male. On the basis of female capitulum, two structures are visible, the areae porosae, which produce a wax coat for eggs, protecting them from drying. Pictures were adapted from the website https://www.flickr.com/ and taken with magnitude of about 200X.

Chelicerae are relatively large and short-shafted, surrounded by a tough, spinose sheath and characterised by laterally directed cutting edges. They are made up of 2-segmented digits, in which the outer (or external) segment resides in a cavity of the inner one. They are located on the dorsal side of the capitulum and move together, with robust tendons allowing their attachment to the powerful muscles of the bulbous cheliceral basis. Differently from the other parasites belonging to the superorder Parasitiformes (which are able to clamp, grasp, pierce and shear), chelicerae cause a ripping and tearing action against the host skin. The inner digit of chelicerae is also equipped with receptors sensitive to mechanical and chemical stimuli, in order to sense the strength of tearing and the composition of host fluids. They also have an important role in the recognition of sex pheromones (deriving from sterol and steryl ester metabolism) of conspecific females. These pheromones are fundamental in the processes of mounting and copulation, as demonstrated in males of *Dermacentor variabilis* and *D. andersoni* [41, 42]

Palps are the remaining appendages located on the capitulum. They have a structure similar to legs and are characterized by four distinct segments, also called 'articles'. Contrary to legs, palps have only sensory function and do not participate in attachment to host. The terminal segment (article IV) is short and is within a ventral cavity of the third article, and is thought to

be a well-developed sensory area, due to the presence of numerous small setiform sensilla on the blunt end and larger setiform mechanosensory setae along the lateral edges. The other segments bear more long and stout setae, as a protection of the delicate mouthpart. Differently from many gamasid mites, they are lacking of subterminal tined setae or the terminal apotele (pre-tarsus), important structure presents in the walking legs [3].

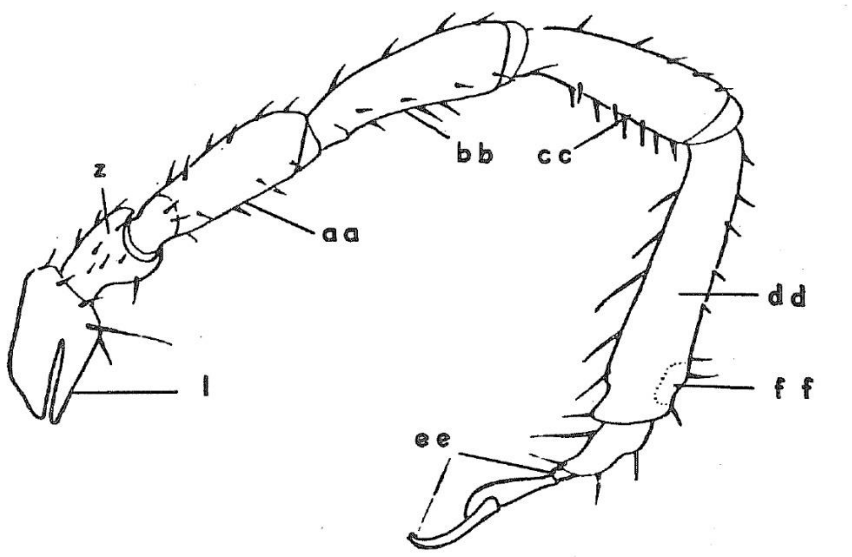


Figure 4. Representative structure of the leg I of Hyalomma asiaticum *female. Abbreviations: l (coxae); z (trochanter); aa (femur); bb (metatarsus); cc (tibia); dd (tarsus); ee (pulvillus and claws); ff (Haller's organ). Adapted from Balashov (1972) [1].*

The most proximal structure of the capitulum is the basis capituli. In ixodid females, its dorsal surface is characterised by a pair of depressions bearing two porose areas, each characterised by tiny pores. Sometimes these areas can cover all the entire surface [3].

The idiosoma

As previously mentioned, the idiosoma is composed of two regions: podosoma and opisthosoma, which bear important taxonomical features, such as spiracles, anus, grooves, plates and other elements. On the ventral side, the podosoma is quite similar in all ticks and bears the legs. Ticks have four pairs of legs similar to many other acarines, each divided into 6 segments (in order: coxa, trochanter, femur, tibia, metatarsus and tarsus). Each segment is joined with the next through a soft articulation cuticle. Coxae are the first segment, directly inserted into the ventral side of the ticks' body. They allow limited movements to legs because of their position, as antero-posterior rotation and raise-lower movements. In males of some species, the coxae are enlarged and cover the most of the ventral surface, offering important

taxonomical features. The other segments are highly flexible, and able to perform flexions (for body protection) and extensions (for walking). The last segment of each leg, the tarsus, ends with an apotele, which includes also a pair of claws and the pulvillus. The dorsal surface of the tarsus of leg I bears a complex and important sensory apparatus, Haller's organ, implicated in several processes, including determination of host odours and location, as well as detecting of pheromones and other stimuli [1, 3].

Dorsally, the idiosoma is different among families. Argasidae have a very flatten body, with a lateral suture markedly dividing the dorsal surface from the ventral one, even if not always evident. The presence of small rectangular plates may occur in the lateral body margin. The body of ticks belonging to this family is characterised by a complex system of grooves, even in the supracoxal region, allowing body enlargement during feeding, and tiny elevations, termed mammillae. Moreover, they are covered by extensible cuticle and small discs on the dorsal surface represent the hard cuticle where muscle are attached. E.g. the grooves system is more evident in the genus *Ornithodoros* and less so in the genus *Argas*, which instead has a more developed hard cuticle. Furthermore, a hard cuticle is present only in larval stages of the genus *Argas* and in some species of the genus *Ornithodoros*, and which disappears during moulting to nymph and adult [1]. Some species belonging to this family show eye-like structures, similar to the ocelli of insects, located on the supracoxal folds. This region also comprises paired coxal pores (i.e. opening of the coxal glands) and spiracles, differently located. The firsts have been identified in the region delimitated by coxae I and II, while the second lay between the coxae III and IV and are smaller compared to those of ixodids. In males, the genital aperture has a lateral orientation and lacks of genital cover. Also in females, the genital pore has a horizontal slit and surrounded by a fusiform cuticolar fold. The anus is surrounded by a short pre-anal groove anteriorly and a T-shaped post-anal grooves posteriorly [1, 3].

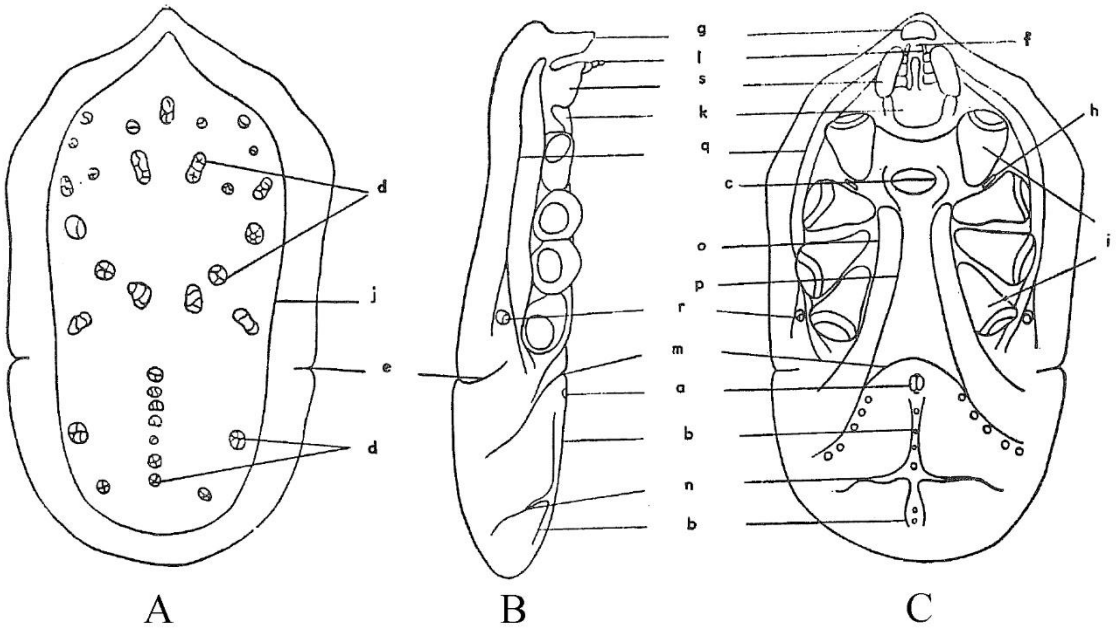


*Figure 5. External anatomy of a representative female argasid tick (*Ornithodoros papillipes*). A (dorsal view), B (lateral view), and C (ventral view). Abbreviations: a (anus); b (anomarginal groove); c (genital slit); d (discs); e (dorsoventral groove); f (camerostome); g (hood); h (coxal pore); i (coxae); j (marginal groove); k (basis capituli); l (palpi); m (tranvers preanal groove); n (tranverse postanal groove); o (subcoxal lateral groove); p (subcoxal median groove); q (supracoxal groove); r (spiracle); s (cheeks). Adapted from Balashov (1972) [1].*

Ixodidae have a flattened body when unfed but expands after feeding. Body structure shows differences in male and female ticks. In males, the ventral surface of the podosoma is characterised of considerable area of extensible cuticle, which is covered by a group of separate and identifiable plates, including the pregenital plate, which is mobile. On the dorsal side, a large and sclerotized plate, called the "scutum", covers the entire body surface, restricting the extensible cuticle to a narrow lateral zone.

Females show an antero-dorsal orientation of the U- or V-shaped genital aperture (taxonomically relevant) on the ventral side of podosoma. The most important difference compared to male is in the dorsal side, because unfed females have a smaller scutum, covering about one third of the dorsal surface. The posterior part is known as alloscutum and lacks sclerities. It is characterised by numerous fine striations, due to a heavy folding of the cuticular surface, as well as evident tiny pores (also in the scutum of males, but not in the genus *Ixodes*) implicated in pheromone secretion and mating behaviour. Grooves and ridges are present on the outer layer of scutum, known as cervical grooves and lateral carinae, respectively. Moreover, the surface of scutum is commonly smooth but highly punctate (i.e. pitted) in most species, but a rugose pattern or the presence of few small short setae may occur [1, 3]. In

addition, minute ear-like pores, sensilla auriformia, may be found on the surface of scutum and alloscutum. These are proprioreceptors responding to pressure changes in the cuticle. Moreover, many species bear eye-like structures located on the lateral margin of the scutum. Females also have a cavity between the scutum and the basis capituli, which is occupied by the Gene's organ, which has an important role in the oviposition. Spiracles, which are arranged on the large and elongated spiracular plate, are located laterally and behind the coxae of legs IV. The spiracular plate is covered by semitrasparent goblets, whose number and arrangement are useful for species identification. The anus is located in the anal groove of the opisthosoma region, posteriorly and medially to the spiracular plate. It is prominent in the genus *Ixodes* (typically U-shaped, extending from the posterior margin of the body to a region anterior to the anus), and smaller in the other genera. festoons, small cuticular areas separated by short grooves, are present in the opisthosoma region in all Ixodidae, except for the genus *Ixodes* [1, 3].

Figure 6. Dorsal (left) and ventral (right) aspect of a male Dermacentor. *This species is characterised by an ornamented scutum, and coxae (from 1 to 4) increasing in size. In the ventral side of tick is possible to notice the festoons (black arrow) Adapted from Bowman (2014) [2].*

Integument

The most external portion of the tick body is termed integument. It plays an important role in protection against water loss, but also against mechanical and other physical damage. It consists in a single layer of epidermal cells, which secrete the outer layer of the cuticle thus forming the exoskeleton. The integument also comprises the various cuticular appendages, such as setae,

spines, spurs, discs, and plates. The cuticle is the dead part of the integument and consists of two main layers, the thin external epicuticle and the thicker internal procuticle, composed of macromolecular glycoprotein micelles and chitin. Glycoproteins and chitin are essential for the development of the sclerotized cuticle (i.e. scutum) in the outer region of procuticle, termed exocuticle. In ixodid females, nymphs and larvae, the epicuticle (and the relative underlying procuticle) of extendible areas is folded in regular rows, which are characteristic for each species. Feeding activates the inner epidermal layer, expanding greatly the procuticle, which synthesizes new cuticle. In juvenile stages, this is essential for the synthesis of several hormones activating the ecdysis process. In addition, in females of *Ixodes ricinus*, the procuticle prepares for rapid engorgement increasing its weight of about 20 times, even if its thickness remains unchanged because of stretching. Most of this increase occurs during the first seven days after attachment. In other species (i.e. *Dermacentor variabilis*), procuticle growth is gradual during feeding and almost zero during the rapid repletion phase [43].

All cuticle layers and the integument (i.e. the epidermis) offer a fundamental barrier against water loss in ticks. The lipid layer in the outer surface of cuticle forms a waxy coat, crucial for survival, distribution and activity of ticks [44-47]. However, different species respond to water loss in different ways, because of their own critical temperature, which induces a dissociation of the wax molecules and an increase in permeability to water (e.g. *I. ricinus* loses water 10-15 times more rapidly than *Dermacentor andersoni*) [48]. Salivary glands also contribute to this purpose, secreting and imbibing hygroscopic substances that actively absorb water from unsaturated air [45].

The inner body of ticks consists in a hemocoel (an open cavity) where organs and apparatuses lie. The presence of the fluid hemolymph, as well as loose connective tissues and membranes, support the internal organs.

Musculature

Ixodid ticks have a well-developed musculature, which has been meticulously described in historic anatomical papers [49, 50]. Several dorso-ventral muscle groups have been described, each with their own location. Contraction of muscles allows changes in body size and form, is important for defecation and oviposition, and for the movements of capitulum. Muscle groups of the body are joined to the integument in correspondence of grooves. Leg muscles allow flexion and extension movements and consist of oblique bundles of coxal and subcoxal muscle groups [1, 3].

Digestive apparatus

The efficient system for acquiring and processing host blood is the key to the success of ticks. This system has been studied since the last century, and the digestive system *I. ricinus*, has interested the scientific community since 1861 [51].

The alimentary system is divided into three main regions:

- foregut, which comprises the wide preoral cavity, a pair of salivary glands, the pharynx and the esophagus;
- midgut, including the stomach and lateral diverticula;
- hindgut, which includes the small intestine, rectal sac and rectum, which opens into the anal aperture.

All the organs of this system have origin from embryonic ectoderm or endoderm.

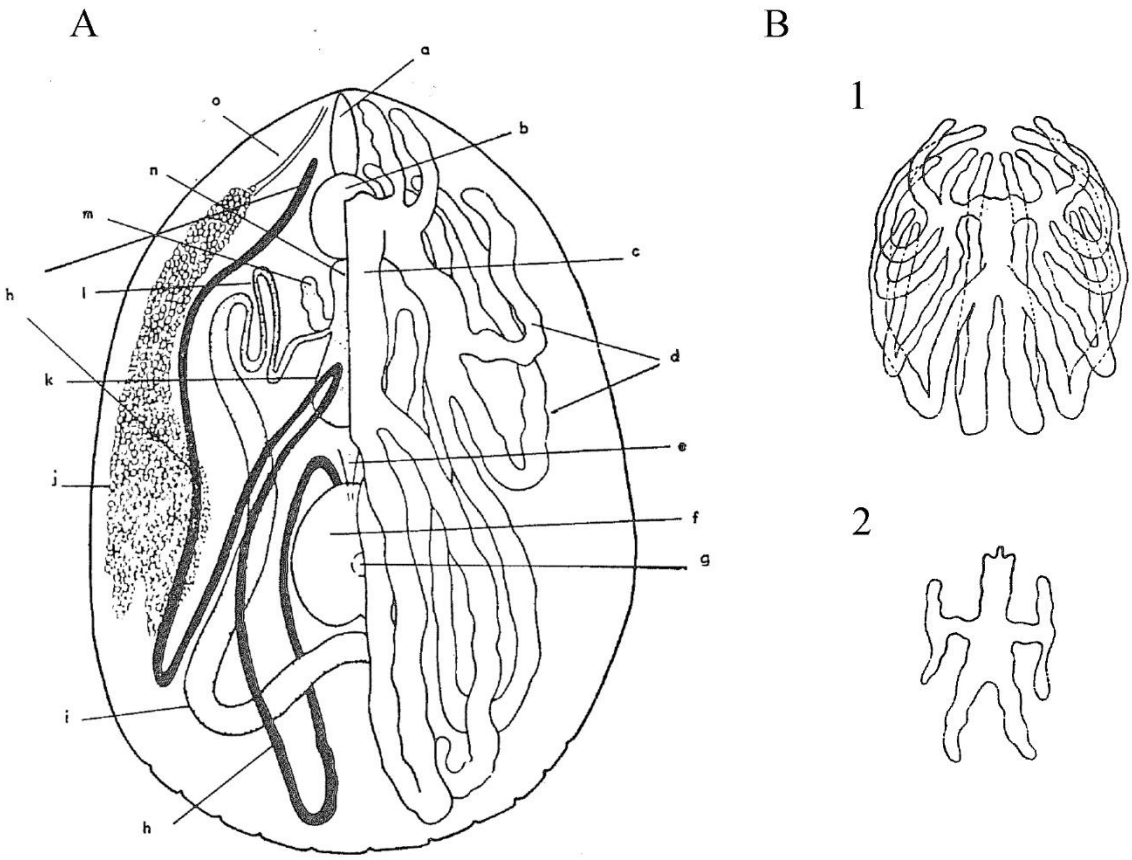


Figure 7. A. Inner morphology of dissected female of Hyalomma asiaticum, *dorsal view. In the left half, the gut was removed, in order to emphasise the structure and disposition of other organs. B. Comparison of midgut ramification in* Ixodes ricinus *female (1) and male (2). Abbreviations: a (pharynx); b (brain); c (gut); d (midgut diverticula); e (small intestine); f (rectal sac); g (anus); h (Malpighian tubule); i (ovary); j (salivary gland); k (receptaculum seminis); l (oviduct); m (accessory gland); n (vagina); o (salivary gland duct). Adapted from Balashov (1972) [1].*

The preoral cavity comprises the mouthpart. As previously described, it bears the chelicerae, which cut and tear the host's skin by lateral movements, allowing the penetration of the hypostome and the preoral canal. After penetration, ticks begin the suction of nutrient fluids by the action of a pharynx, near the basis capituli. The feeding process is simplified by tick saliva, which is responsible for the suppression of host inflammatory and immunological processes [52]. They consist in two grape-like clusters, formed of several types of alveoli attached to greatly branched efferent ducts and connected by loose connective tissue and ramifying tracheae. The size of the salivary glands is different among subfamilies and reflects the physiological state of the ticks, as they are larger in feeding than in unfed ticks. Moreover, salivary glands in ixodid ticks play an important role in excretion of fluids as large volumes of blood are ingested, (while in argasid ticks, for example this function is executed by coxal glands) [53, 54]. Tick saliva contains proteolytic enzymes that digest and liquefy tissues and a cementing material to reinforce the attachment to the host skin. The preoral canal is regulated by an elaborated pharyngeal valve. The pharynx gradually becomes a tube-like, short and thin esophagus, which opens posteriorly into the midgut. The midgut consists of a central stomach and numerous diverticula (caeca), extending along the entire body. The number of caeca vary among species and even among genders; e.g. some caeca are so reduced that only two pairs of transverse processes extend from the elongated tubular stomach, particularly in males of *I. ricinus*, but generally in males of the Subfamily Ixodinae. Tick midgut is characterised by two kinds of epithelial cells: undifferentiated and digestive. The latter develop from the first and food material is ingested (through pinocytosis or endocytosis) and then digested [1, 3]. This is different from other blood-feeding arthropods, which have a rapid extracellular digestion, and is the reason why several pathogens are able to survive in the tick body and spread into other tissues [3]. A tubular small intestine follows (designed only for faecal waste transport, as there is no evidences of digestion in there), gradually narrowing to the rectal sac, where Malpighian tubules are located. These structures are histologically similar to the rectal sac (constituted by a simple cuboidal epithelium). They are long and narrow tubules, looping and twisting among other organs and tissues and keeping in contact with every organ or tissue for the harvesting of nitrogenous waste products. Ticks only have a single pair of tubules, while other insects have a multiple tubule system [4]. Faecal waste of ticks is prevalently constituted of dead or dying cells, cell fragments, hematin (detoxification product of heme), undigested haemoglobin crystals, guanine crystals and other fluids. The waste backflow to the intestine and the midgut is hindered by the presence of a circular muscle surrounding the junction segment b intestine and the rectal sac [1]. The rectal sac opens medioventrally into the rectum, lined by a thick

cuticle, and then the anal aperture. The waste flow is guaranteed by the contraction of the intestinal wall muscles. The contraction of a pair of muscle bundles, extending from the rectum walls to the anal plate, allows the aperture of the anal slit, facilitating defecation.

Excretory organs

In ticks there are two organs implicated in excretory functions. The first are the Malpighian tubules, whose structure has been extensively studied and described above [55, 56]. The histological structure of these tubules consists of a single layer of epithelial cells with differences in shapes. The possible presence of rickettsiae pathogens, which usually localise to the anterior ends of tubules, alters cell morphology and the relative shape of the structures. The second organ implicated in excretory function is the coxal organ. This consists of a pair of porous slit-like aperture, located between coxae I and II, through which ticks discharge a coxal fluid, important for the maintenance of water and salts balance.

Circulatory system

The blood circulatory system of ticks is open, as in the other arthropods and organs are rudimentary. The circulatory fluid is called "hemolymph", which bathes all organs and fills the body cavity and the capitulum. It consists of a mixture of plasma and hemocytes, and the relative percentage of haemocytes versus body weight remains constant, even after feeding. At least five kinds of hemocytes have been recognised, even if this classification is still controversial:

- prohemocytes, small undifferentiated cells, probably giving rise to other hemocytes;
- plasmatocytes, thought to be wandering phagocytic cells;
- granulocytes, involved in processes of hemolymph clotting, as well as in the release of granules responsible for the coagulation and encapsulation of particles or microbes;
- spherulocytes, similar in appearance to granulocytes;
- oenocytoids, containing large cytoplasmic granules and responsible for lipid processing and detoxification [1, 3].

Hemolymph circulation is guaranteed by a small, triangular and dorso-ventrally flattened heart, joined to the dorsal body surface by fine muscles. The heart is surrounded by a pericardial sinus, which acts as a filter. Hemolymph enters the heart through tiny paired ostia, which pump it into a dorsal aorta, pass through the periganglionic sinus (surrounding the synganglion), the pedal arteries and other sinuses to, finally, spread into the basis capituli and the body cavity.

Respiratory system

All the adults and nymphs of the family Ixodida have a tracheal system, through which air flows to the organs and tissues. The air intake is guaranteed by a pair of respiratory pores, termed "ostia", located on the spiracular plates. On the surface of the spiracular plates are visible tiny goblets, separated by pedicels, which is a complex system of tiny air spaces. Below each ostium is a sac-like atrium, connected by up to eight large tracheal trunks to a system of ramifying tracheae and tiny tracheoles spreading throughout the body cavity. Spiracles are provided with a closing mechanisms coordinated by muscles under the control of the nervous system. This complex system is missing from the larvae of all ixodids [1, 3].

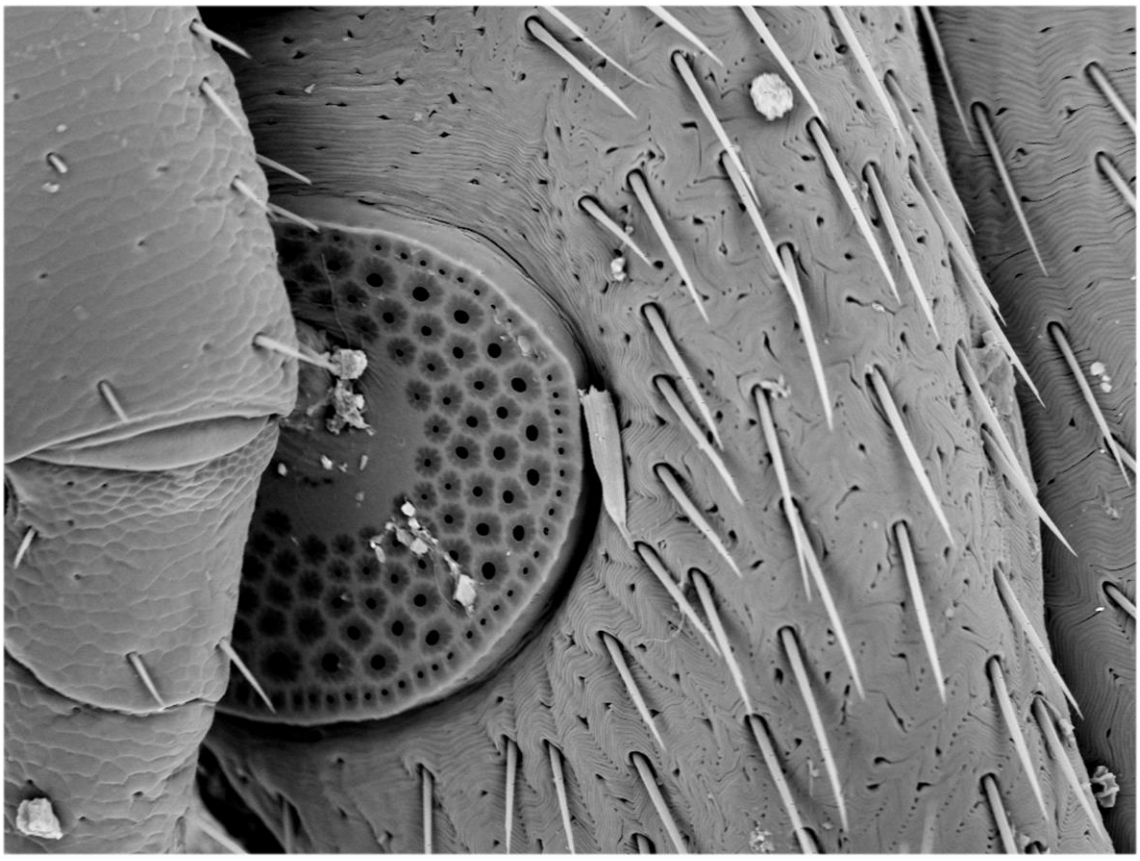


Figure 8. Scanning electron microscopy of Ixodes ricinus*. In particular, the spiracular plate sided in the ventral-lateral portion of the tick body. Pictures were adapted from the website https://www.flickr.com/ and taken with magnitude of 591X.*

Reproductive apparatus

The male genital system of ixodids is similar in all species, with small differences in anatomy, and consists of two tubular testes and paired vas deferens (or vasa deferentia), an ejaculatory duct and an accessory gland complex.

Testes are tubular organs, which become more elongated and prominent during feeding. They appear completely separated in ticks belonging to the genus *Amblyomma*, partially fused in the species *I. ricinus* and fused posteriorly in the family Argasidae. They extend along the lateral margins of the body up to the rectal sac, where they turn and fuse (or joined by a septum) and open in short and coiled vas deferens, which fuse in the next seminal vesicle. The vas are almost imperceptible in unfed males and become more evident during feeding for the presence of numerous spermatids. A short ejaculatory duct connect the vesicle to the genital pore. The gland complex is a multi-lobed system lying upon the midgut diverticula and posteriorly to the brain. It is different in form and size among the families and secrete glycoproteins, mucoproteins, lipoproteins, mucopolysaccharides and proteins important for the capacitation of the sperm.

In females, the reproductive system is similar in both argasid and ixodid ticks and morphological differences are associated with the functional differentiation of the vagina. The system comprises a single ovary, paired oviducts, single uterus, a vagina (subdivided into cervical and vestibular parts) and a large seminal receptacle (or receptaculum seminis, which is absent in Prostriata and Argasidae). A pair of accessory genital glands open in correspondence of the junction between the two vagina portions [1, 3].

In unfed ixodid females, the ovary is U- or horseshoe-shaped and is relatively smaller in the subfamily Ixodinae than in Amblyomminae. Feeding causes an increase in ovary size, which will occupy most of the body cavity at repletion. The ovary is joined at its end with two turning and looping oviducts, which open into the uterus. A connecting tube finally connects the uterus with the cervical vagina (posteriorly muscular and thick-walled), and the vestibular vagina (thin-walled and lined with highly folded cuticle). In the Metastriata, the presence of a seminal receptacle above the cervical vagina allows the storage of sperm introduced during copulation. Ixodid female ticks have a second pair of accessory glands and the Gene's organ, a structure with protruding finger-like extensions, important for waxing eggs during oviposition [1, 3].

Nervous system

The central nervous system is characterised by a single whitish, sub-spherical mass, termed synganglion. This derives from the merge of neurons, surrounded by a thick perineurium, and a complex membrane of glial cells and their processes. It lies dorsally and slightly posterior to the genital pore in adults and in a comparable position in immature stages. The presence of the esophagus divides the mislabelled 'brain' into two regions, the supra- (in an antero-dorsal position) and sub-esophageal (in a postero-ventral position) regions. The small supra-esophageal region consists of protocerebrum, optic centres (reduced in eyeless species),

chelicera ganglia (coalesced in ixodids), the stomodeal pons (or bridge) and the palpal ganglia. A branch of the palpal ganglia is able to innervate the salivary glands [57], while esophagus and pharynx are innerved by an unpaired esophageal nerve from stomodeal pons. The sub-esophageal region is characterised by four pairs of lobe-like pedal ganglia, which give off four pairs of trunk-like pedal nerves on each side of the synganglion, and the unpaired ventral nerve ganglion. Two pairs of nerves (opisthosomal nerves) extend from the last to the internal organs. Several studies, Ioffe (1964) before and others later, confirmed the presence of eighteen neurosecretory centres occurring in the synglanglion [58]. Lobs on the ventral surface receive axons from the Haller's organ, while the sub-esophageal region receives motor neurons, suggesting a primary role in motor coordination. The presence of small segmental organs, which act as endocrine glands, is evident in each plexus.

Tick Life-Cycle

All ticks are oviparous and mature throughout four stages. The first stage is the embryonated egg, followed by three main active stages, represented by a larval stage, one nymphal stage and the last adult stage. Male and female discrimination is possible only in the last stage, with the presence of the genital aperture and of external structures (i.e. the scutum). Ticks mature from one stage to the next through a moulting process, following a blood meal on the host. However, examples of moulting in non-feeding tick stages have been described in argasid ticks: larval and nymphal stages of the genus *Ornithodoros* or adult females of the genera *Otobius* and *Autricola* [1].

Ticks are classified according to the number of hosts on which they feed during their life cycle: one-, two- or three-hosts. One-host ticks complete their cycle upon the same host and then drop off to lay eggs. Two-host ticks complete the juvenile stages upon the same host, then drop off and moult to adult, which then infects a second host for the last meal. Three-host ticks use different hosts for each stage of their life cycle. After the last blood meal, adult engorged ticks drop off the host and search for a niche to lay eggs (typically 2000-10000 eggs, but their number can exceed 22000, as reported by Arthur (1962) in *Amblyomma nuttalli*). The duration and phenology of the different motile life stages vary with species, geographical location, relationship to hosts, as well as environmental conditions, i.e. temperature, relative humidity and number of hours of daylight to which they are exposed. These factors influence the length of the tick life cycle [1, 3, 53]. In some species, it can take even less than one year. Furthermore, optimization of environmental conditions (i.e. laboratory conditions) can considerably reduce the period between two consecutive generations. E.g. *Hyalomma impeltatum* completes its cycle in about 108 days when the laboratory conditions are set to 26 ± 1°C, high relative humidity and a 12:12 photoperiod [59]. However, climatic conditions are not always favourable and diapause may occur, extending the entire life cycle till more than three years, as described in *I. ricinus* [48].

Based on their habits, ticks may be classified as nest-dwelling (nidicolous) or field-dwelling (non-nidicolous) parasites. Nidicolous ticks are almost all argasid ticks and some ixodid ticks, such as those belonging to the genus *Ixodes*, which spend nearly all their life off host. They find sheltered environments in rock ledges, crevices or burrows, nests, caves, or hide in soil cracks of tree bark or wood nearby host-occupied sheltered sites, which offer stable microclimates (temperature, relative humidity and wind) and ideal conditions for tick development, reproduction and host seeking. Non-nidicolous ixodid ticks have limited tolerance to

desiccation but compensate by moving vertically on vegetation, according to host needs and humidity (although they have the ability to absorb moisture directly from the air). They may be found in varied environments, such as forests, savannahs, scrub, moorlands, pastures, meadows, brush and even desert [1, 3, 53].

Ixodidae

The life cycle is the same in all Ixodidae ticks and characterised by a single nymphal stage. As mentioned above, female feeding causes an increase in body weight of more than one hundred times versus the unfed one. Cholesterol and esteryl methabolism induces the release of sex pheromones, which favour male and female mating. Copulation usually takes place on the host, except for ticks of the genus *Ixodes*. After repletion, females drop off and begin to lay eggs (e.g. under leaf litter, rotting vegetation, cracks or crevices in natural or man-made shelter). Gonotrophic cycles (i.e. egg production and oviposition) increase gradually during the first 3 to 5 days and, after 10 days, females lay the 90% of the egg mass, continuing for further 5-10 days to its complete emptying and death [3].

Three-host life cycle

Three-host life cycle is the most complex and it is characterised by several phases of seeking hosts and feeding for each of life stage. Usually, juvenile stages prefer small hosts to parasitize and complete their blood meal on average 2.5 to 8 days, whereas adults prefer big ones and are completely engorged in 5 to 12 days [1, 3, 48]. The entire cycle has variable duration, depending on several climatic and can be completed even in less than one year.

Hatching begins a few days after oviposition, and emerging larvae respond to environmental condition, adopting a questing behaviour or entering diapause, if conditions are not favourable [60]. In the presence of appropriate conditions of temperature and relative humidity, they adopt questing behaviours, which lead larvae moving into the vegetation or nest environment to seek hosts. Successful finders attach to the host, move on and fix the mouthpart into the skin. Blood sucking is completed in few days, than larvae drop off the host, move into a sheltered microenvironment and undergo ecdysis. The emerging nymphs respond to environmental conditions, activating the questing behaviour or the behavioural diapause. Questing nymphs move to seek hosts (often the same of the previous step) and complete the blood meal again in few days. Engorged nymphs drop off the hosts and moult in adults (males or females), which emerge, seek for the third hosts, feed and mate (in the genus *Ixodes* mating can occur even

before feeding). Engorged female drop off the host, move in sheltered microenvironment and lay eggs or enter an ovipositional diapause even for several months. The ovipositing female lays numerous eggs and then dies.

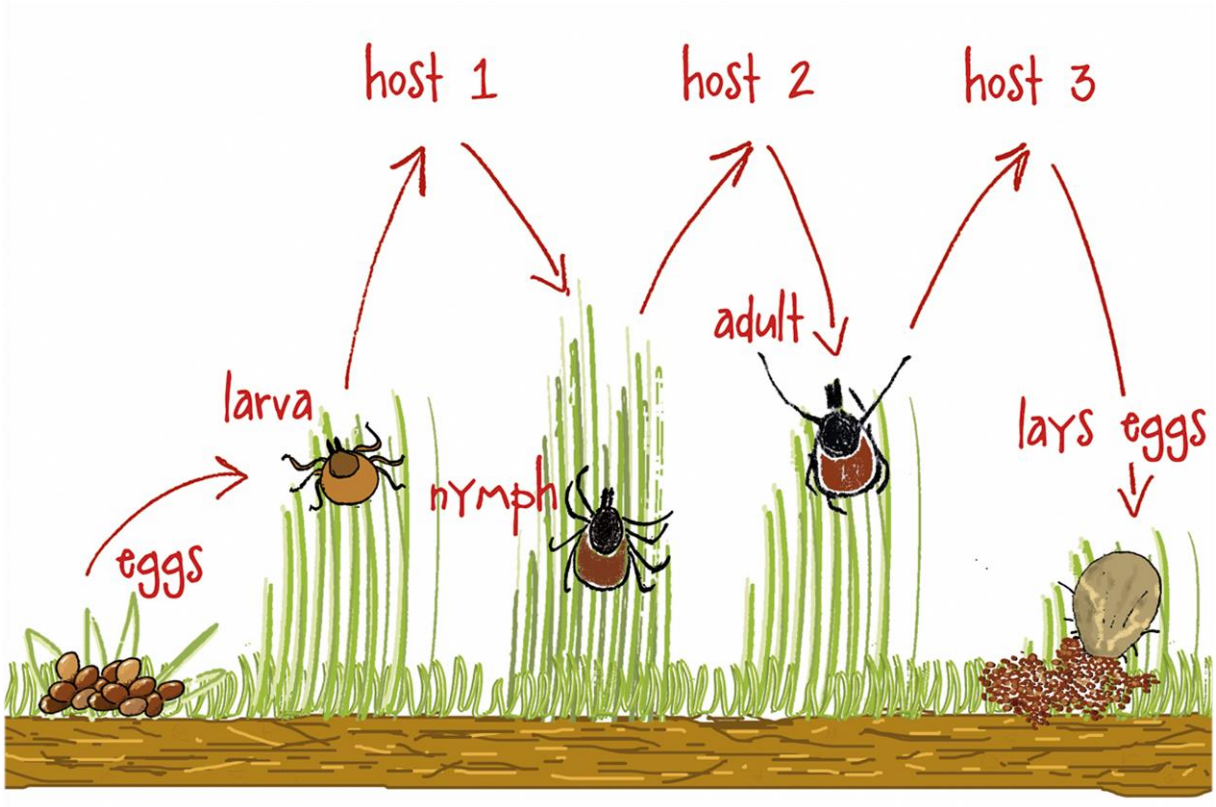


Figure 9. Schematic representation of the Ixodes ricinus *life cycle. It is a typical three-host tick, which completes its cycle within three (or more) years. The drawing was a kind gift from Prof. Marco Genchi.*

Tick distribution and importance

Ticks are widely spread around the world, from the tropics to subarctic areas. The greatest diversity is present in the tropical and subtropical regions, due to their optimal climatic conditions [1, 3]. However, ticks are widely present even in Europe, and the prevalent genera and species recognised are reported in the table below.

Table 1. Major tick genera and species spread throughout Europe.

Genus	Species
Ixodes spp.	*I. ricinus*
	I. canisuga
	I. hexagonus
	I. persulcatus
Rhipicephalus spp.	*R. sanguineus*
	R. bursa
	R. turanicus
	R. pusillus
Dermacentor spp.	*D. reticulatus*
	D. marginatus
Haemaphysalis spp.	*H. punctata*
	H. concinna

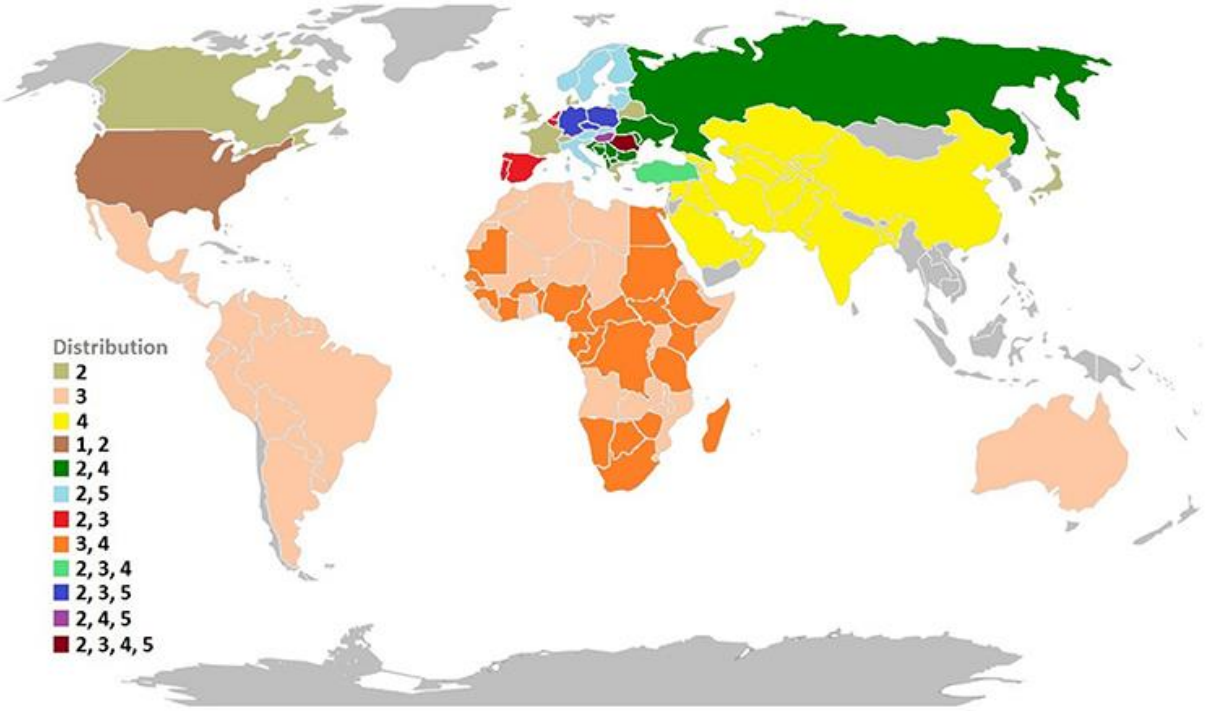
The importance of tick distribution is certainly due to their direct effects on the host, with phenomena of blood loss, direct toxicosis, and secondary infections. As reported by Koch and Sauer in 1984, each replete female is able to ingest at least 0.5 ml of host blood, with peak of 1.45 ml for *Dermacentor variabilis* [61]. Tick bites can cause deep and painful wounds in the host skin, which tend to become inflamed and can be secondarly infected with bacteria, and flyblown. In Great Britain, for example, secondary infection of *Ixodes ricinus* bites with *Staphylococcus* results in local and metastatic abscessation in lamb [2]. Ticks engorge a considerable volume of blood and inject the surplus water extracted back into the host. This function is complemented by the salivary glands, which produce many diverse substances.

Among these, toxins have been identified [62]. A single female tick can induce paralysis in humans, dogs, or cats, especially if the site of attachment is near or on the head, but paralysis does not invariably occur even if many ticks of a species are present. In cattle, for example, tick paralysis is induced by heavy infestations. The last phase of tick paralysis affects the respiratory muscles, with fatal consequences. Removal of engorging females usually leads to a rapid recovery [2].

The major interest in understanding and controlling ticks is due to their potential vector role. In fact, ticks transmit a wide variety of pathogens to vertebrates including viruses, bacteria, protozoa and helminths (Table 2). Ticks are considered to be second worldwide to mosquitoes as vectors of human diseases, but they are the most important vectors of disease-causing pathogens in domestic and wild animals, and are believed to be responsible for more than 100,000 cases of illness in humans throughout the world [7].

Table 2. Representative examples of the different categories of tick-borne diseases of humans, and domestic or companion animals. For each disease the principal vector(s) and host(s) are reported the principal vector(s) and host(s). The table was adapted from Sonenshine (2014) and Deplazes (2016) [4, 5].

Category of causative agent	Representative disease	Causative agent	Major tick vector(s)	Primary host(s)
Protozoa	Babesiosis	*Babesia bigemina, B.microti, etc*	*Rhipicephalus* spp.	Cattle, deer
	East Coast fever	*Theileria parva*	*Rhipicephalus appendiculatus*	Cattle, cape buffalo
	Theileriosis	*T.annulata*	*Hyalomma* spp.	Cattle, water buffalo
	Human babesiosis	*B.microti*	*Ixodes scapularis*, *I.ricinus*	Humans, mice
	RMSF	*Rickettsia rickettsii*	*Dermacentor* spp.	Humans, dogs, small mammals
	Mediterranean spotted fever	*R.conorii*	*Rhipicephalus sanguineus*	Humans, hedgehogs, small mammals
	Anaplasmosis	*Anaplasma phagocytophilium*	*Ixodes scapularis*, *I.ricinus*, others	Humans, deer, dogs, others
		Anaplasma marginale, A. centrale	*Dermacentor* spp., others	Cattle, sheep, other ruminants
Bacteria	Human ehrlichiosis	*Ehrlichia chafeensis*	*Amblyomma americanum*	Humans, deer
	Canine ehrlichiosis	*E. canis, E. ewingii*	*R. sanguineus*, others	Dogs
	Heartwater	*E. ruminantium*	*A. hebraeum, A variegatum*	Cattle, other ruminants
	Q fever	*Coxiella burneti*	Various species	Cattle, humans, other mammals
	Lyme disease/lyme borreliosis	*Borrelia burgdorferi*, other *Borrelia* spp.	*I. scapularis*, *I.ricinus*, others	Humans, diverse mammals, birds
	Tick-borne relapsing fever	*Borrelia* spp.	Various argasid spp.	Humans, other mammals
	Tularemia	*Francisella tularense*	*Haemaphysalis leporis-palustri*, *Dermacentor* spp., others	Lagomorphs, humans, other mammals
	Tick-borne encephalitis	*Flavivirus*	*I.ricinus*, other *Ixodes* spp.	Rodents, humans, other mammals
	Colorado tick fever	*Coltivirus*	*D. andersoni*	Humans, various mammals
Virus	Crimean-Congo hemorragic fever	*Nairovirus*	*Hyalomma* spp.	Humans, lagomorphs, hedgehogs, etc.
	African swine fever	*Iridovirus*	*Ornithodoros porcinus*	Pigs, warthogs
Fungus (*Actinomycetales*)	Dermatophilosis	*Dermatophilosus congolensis*	Tick-associated; no proven transmission	Cattle, other domestic animals
Helminths	Rodent filariosis	*Acanthocheilonema viteae*	*Ornithodoros moubata*	*Meriones*
	Apathogenic	*Cercopithifilaria grassii*	*R. sanguineus*	Dogs
	Apathogenic	*Dipetalonema dracunculoides*	*R. sanguineus*	Dogs, foxes, hyenas
	Filariosis of snakes	*Macdonaldius oschei*	*Ornithodoros talaje, O. puertoricensis*	Snakes
Tick	Tick paralysis	Tick-transmitted proteins	Many tick species	Humans, cattle, other domestic animals, birds, etc.
	Tick toxicoses, tick-bite allergies	Tick-transmitted proteins	Many tick species	Humans, cattle, sheep, other mammals, birds



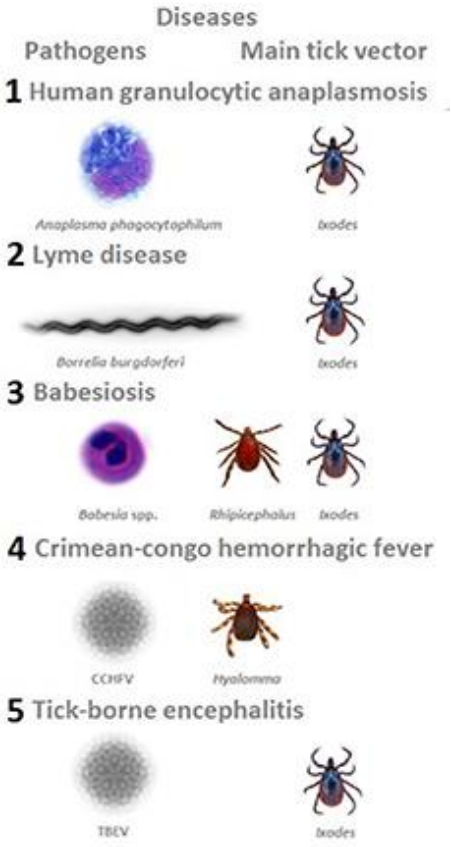


*Figure 10. Distribution model of several tick-borne pathogens and main tick vectors. It includes bacteria (*A. phagocytophilum *and* B. burgdorferi*), viruses (Crimean-Congo hemorrhagic fever virus, tick-borne encephalitis virus), and protozoa (*Babesia *spp.) transmitted by ixodid ticks. Adapted from de la Fuente* et al. (2017) [63].

Ixodes ricinus

The sheep tick, *Ixodes ricinus* is the most abundant and widespread tick that occurs in the vast area of Eurasia, within 39° to 65° north latitude and extending from Great Britain easterly to 55° to 55° longitude, where it overlaps with *Ixodes persulcatus* [53, 64].

I. ricinus is a three-host tick and the life cycle, as previously described, is very complex. It has been collected from at least 300 different species of birds, mammals, and lizards. Larvae and nymphs feed on small mammals, lizards, and ground-inhabiting birds, but adults feed only on large mammals, such as deer and livestock.

The parasitic phase of *I. ricinus* on its hosts is limited to few days, according to the developmental stage. Larvae spend up to five days on vertebrate hosts, while nymphs and adults require up to seven and eleven days, respectively. Moreover, each stage requires its own specific microhabitat comprising various biotic and abiotic factors. Despite these features that make *I. ricinus* extremely vulnerable to alterations in habitat structure and availability of vertebrate hosts, its distribution area has significantly expanded over the past decades, spreading in more northern areas and higher altitudes [10, 11, 15, 65]. *I. ricinus* diffusion can be observed comparing two ECDC (European Centre for Disease Prevention and Control) reports, published in 2012 (Figure 11) and in 2017 (Figure 12).

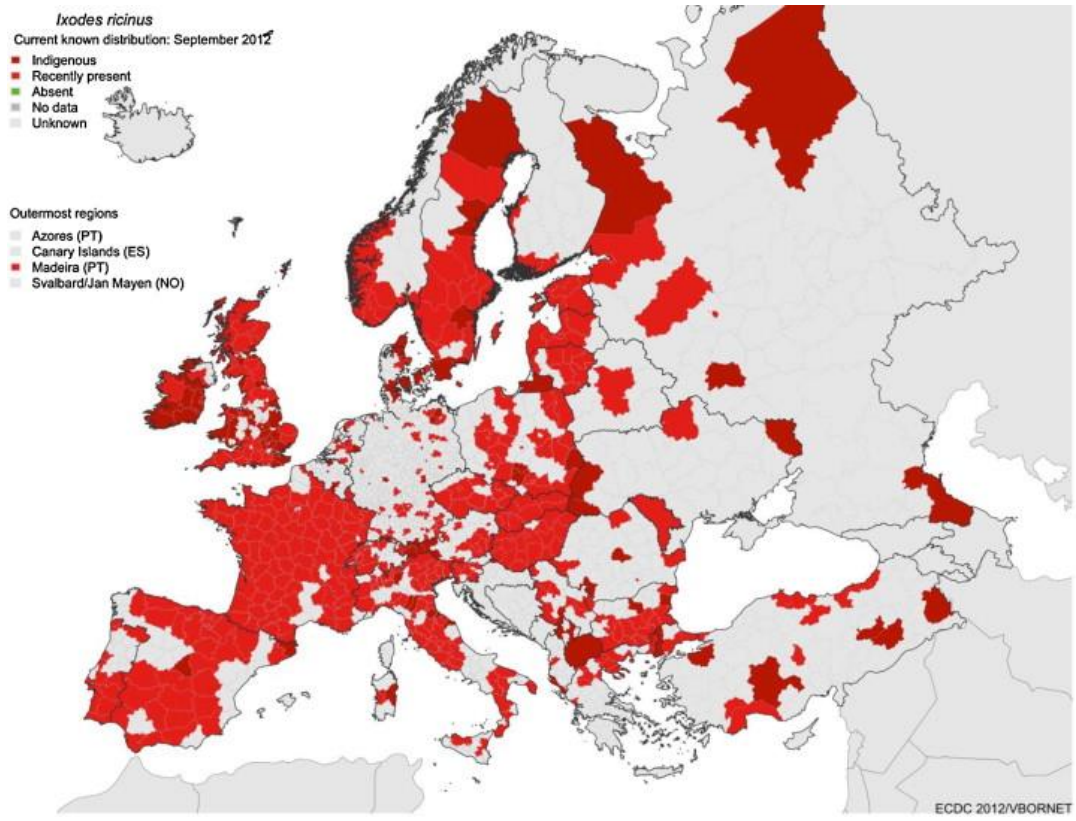


Figure 11. European Centre for Disease Prevention and Control (ECDC) report on Ixodes ricinus *distribution in Europe in 2012, as reported by Beugnet and Chalvet-Monfray in 2013[66].*

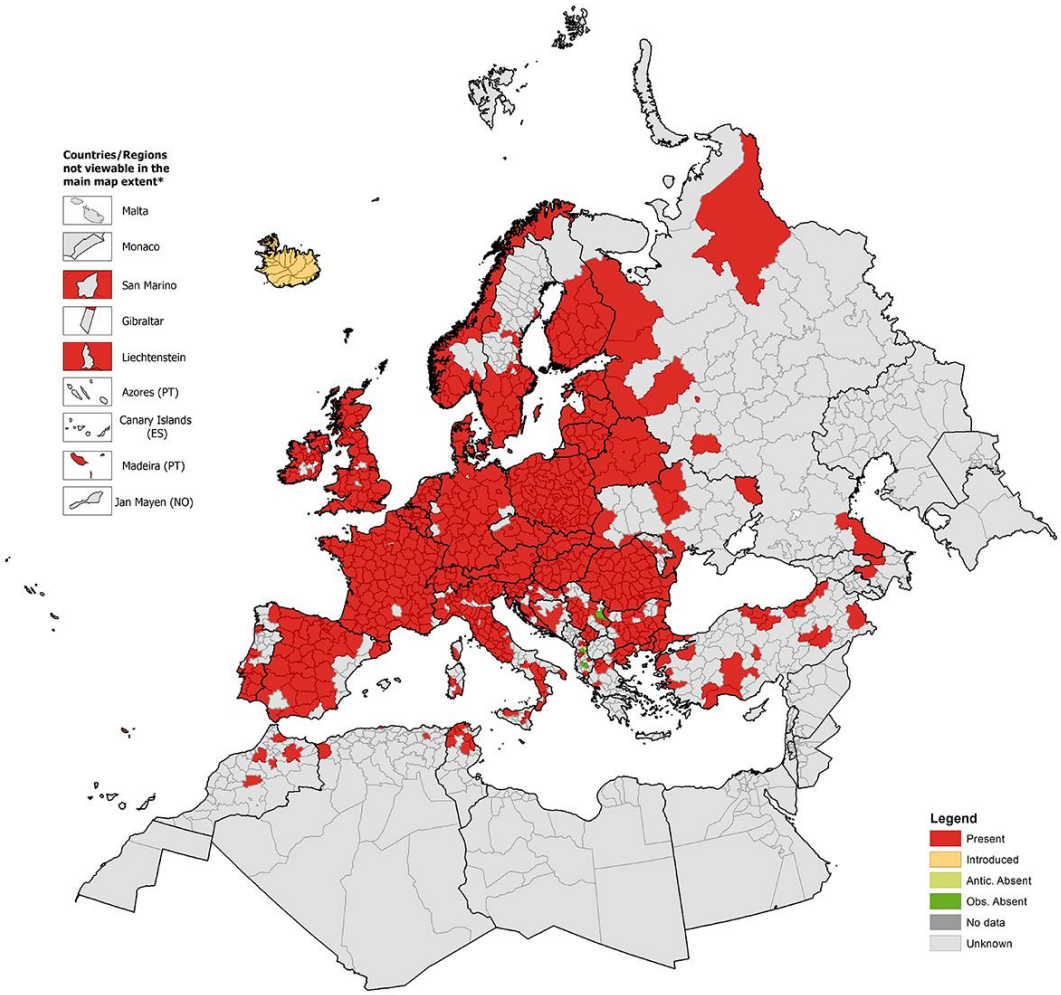


Figure 12. Latest ECDC report on Ixodes ricinus *distribution in Europe (28 April 2017). Available on the European Centre for Disease Prevention and Control website (https://ecdc.europa.eu/en).*

Differences in both reports might be the result of increasing number of publications due to higher awareness in problems related to tick diffusion. However, their spreading is certainly a direct implication of the global climate change [10, 11, 65] and several studies have been carried out to predict environmental modelling and projections of climate niche for ticks, in particular for *I. ricinus* [8, 9].

The increasing interest in *I. ricinus* monitoring relies in another important consideration on tick diffusion. Although the favourite habitat is represented by dense vegetation within shrubs and deciduous and mixed forests, socio-demographic factors, agricultural and wildlife management, deforestation and reforestation allowed the presence of large animals like deer and wild boar in city parks, and in peri-urban areas in many European countries. The direct consequence of all the previous factors is the spreading and stabilising of ticks in the same sites [13]. Moreover, the high abundance of small, medium, and large wild vertebrate hosts in the natural environment was balanced by a shift in the tick–host associations to smaller animals, such as hedgehogs,

foxes, hares, domestic dogs, or cats. The final result of all these considerations is the increasing risk of pathogen introduction and circulation, with consequent health issues for humans and domestic animals [12, 13]. Higher prevalence of infections with tick-borne pathogens have already been observed in the Slovak Republic and in some regions of the USA [67, 68].

There is a real concern that tick spread will be the cause of pathogen appearing in areas previously free of such diseases. Therefore, the beginning of an epidemiological surveillance of the hosted parasitic communities (and potentially transmitted by ticks) is fundamental, in order to preserve human and animal health [64, 69, 70].

Tick-borne encephalitis virus

Tick-borne encephalitis is caused by the TBE virus (TBEV, Flaviviridae), and it is the most important arbovirus transmitted to humans in Europe and eastern and central Asia, although two-thirds of TBE infections in human are asymptomatic [71]. It is currently endemic in 27 European countries [72], with evidences of its expansion into higher altitudes and latitudes, facilitated by the climatic changing previously described [67, 73].

TBEV infection rate in ticks is usually less than 1% of the whole population, but the percentage can increase up to 15% in active microfoci [74-77]. However, the prevalence of infected ticks was observed to be more than 1% even in a highly urbanised region of Southern Poland [78].

Ixodes ricinus is the principal vector for the western European (TBEV-Eur) subtype of the virus [79, 80]. Tick-borne encephalitis virus circulates mainly in wild environments, involving vector ticks and reservoir hosts, but economic factors, as well as human habits, are increasing the risk of infection, which can be acquired through bites of infected ticks, but even through the consumption of raw milk of infected goats, sheep, or cattle, or unpasteurized dairy products [81-83].

***Borrelia burgdorferi* sensu lato**

Borrelia burgdorferi s.l. is a bacterial species of the spirochete class, and is the causative agent of Lyme borreliosis. It is present throughout Europe, and in the northern areas of America, Africa and Asia, representing the most abundant tick-borne disease of humans worldwide. The importance of Lyme borreliosis increased during the last decades, because of its threat to the global public health [84]. To date, *B. burgdorferi* is well characterised, concerning genetics, phylogenetic diversity, molecular biology, mechanisms of interaction with hosts, and pathogenicity for humans and other vertebrate species. All this information led to the development of preventive measures, which include even a vaccine.

Of the 19 *Borrelia* species, which composes the *B. burgdorferi* s.l. complex, only three are proven to be responsible for localized, disseminated, and chronic manifestations of Lyme borreliosis in Europe. They are *B. afzelii*, *B. burgdorferi*, and *B. garinii*. Among the other species of medical and veterinary importance, *B. spielmanii* has been detected in early skin disease, while *B. bissettii* and *B. valaisiana* have been detected in samples from single cases of Lyme borreliosis [85, 86]. The clinical role of *B. lusitaniae* remains to be clarified [13].

However, no further data are available on the risk of infection in urban and peri-urban areas, but it is presumably connected with the geographical distribution and spreading of its vectors, *I. ricinus* and *I. persulcatus* in Europe, and with the presence of suitable hosts, such as dogs and cats. Human recreational activities in forested and urban areas increase the risk for contact with infected ticks [13].

The main reservoirs of the spirochete are small rodents and rats, squirrels, dogs, cats, and ground-foraging birds [87-100]. The role of lizards is still controversial [101-103].

Analysis of *I. ricinus* ticks collected in urban parks, gardens, or suburban habitats shows that infection rate is approximately the same as in ticks living in forests. Therefore, the risk of contracting Lyme borreliosis in urban areas could be as high as in natural environment [13].

Anaplasma phagocytophilum

Anaplasma phagocytophilum is a small, Gram-negative alpha-Proteobacterium, which infects mammals. It is an obligate intracellular parasite, with predilection for cells of the immune system, such as neutrophilic and eosinophilic granulocytes, and monocytes. It enters the cells forming a vacuole with the cytoplasmic membrane. Within the vacuole, the bacterium alters the cell metabolism, and begins its replication. In Europe and in the rest of the Northern hemisphere, the main vector of *A. phagocytophilum* is *I. ricinus* [104].

Well known as a disease of ruminants and horses since the ‘60s, the first human case was reported only in the middle of ‘90s, and subsequently was reported in dogs and cats [105-109]. Initially classified in the genus *Ehrlichia,* it was included in the genus *Anaplasma*, based on sequence analysis of the 16S rRNA [110].

Questing ticks harbouring *A. phagocytophilum* have been found in nearly thirty countries in Europe. Almost one hundred cases of anaplasmosis have been described in humans along the European countries, while the seroprevalence rates are highly variable, reaching peaks of 20%. Indeed, the incidence in the USA in 2010 was 6.1 cases per million inhabitants. Different values are certainly due to several factors, such as anamnesis, tick exposure, and age of the patients, together with the occurrence of less virulent strains in Europe compared to those in USA [111].

Mammalian hosts are represented by wild and small ruminants, but even reptiles have been found infected and are important in the pathogen transmission [104].

As reported by Rizzoli *et al.* in 2014, prevalence of *A. phagocytophilum* in ticks collected from urban and peri-urban areas is influenced by seasonal and geographic variability. Although these values reached high percentage (14.5% in Poland), they did not take into account the presence of less pathogenic strains to humans [13].

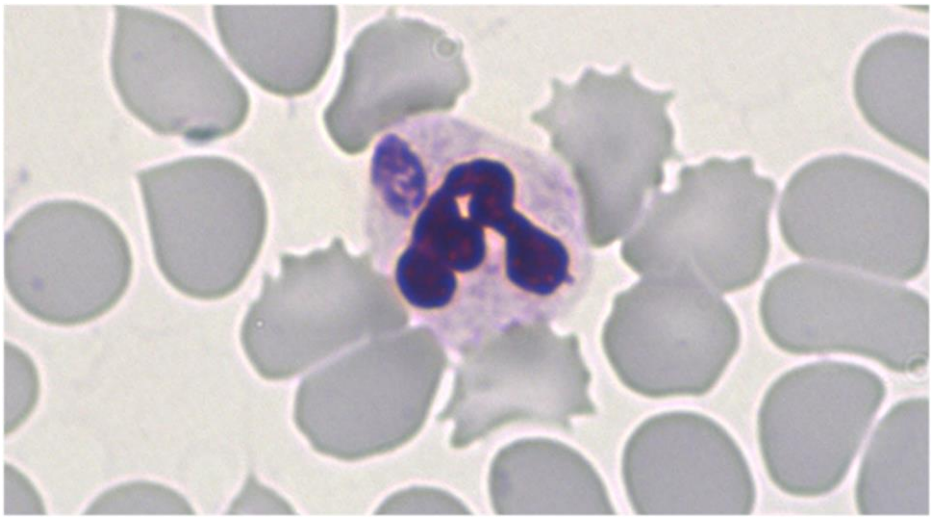


Figure 13. Peripheral blood smear of A. phagocytophilum*-infected host. The pathogen has selective tropism for neutrophils. Magnification 1000x (oil immersion). Picture was a kind gift from Prof. Marco Genchi.*

***Candidatus* Neoehrlichia mikurensis**

Candidatus Neoehrlichia mikurensis is an obligate intracellular Gram-negative bacterium, which is characterised by a selective tropism for endothelial cells. Detected in *Ixodes ovatus*, *I. persulcatus*, and *Haemaphysalis concinna* ticks in Asia, it has been prevalently identified in *I. ricinus* throughout Europe [112-116].

Initially included in the family *Ehrlichia* after its detection in engorged ticks that fed on asymptomatic patients from Italy, it was moved to its currently valid taxonomic denomination "*Candidatus* Neoehrlichia mikurensis" after investigations on the ultrastructure and phylogenetic analysis [112, 117].

Infected ticks have been collected in Italy, Germany, France, Austria, Denmark, Sweden, Russia, and other European countries [114, 118-122]. Prevalence usually ranged between 1 and 11% in sylvatic, urban and peri-urban areas, with peaks up to almost 27% in some regions of Germany [116].

Until few years ago, the detection of *Candidatus* N. mikurensis in ticks or in mammalian hosts was considered only a casual finding without any medical importance [123]. Recently, it has

been linked to unspecific symptoms such as fever, septicemia, malaise, and weight loss in humans with immune deficiency or other primary diseases, and to erythema migrans in 10% of symptomatic tick-bitten adults in the southern part of Norway [124-127]. Moreover, *Candidatus* N. mikurensis infection has been reported in 7 out of 622 patients from China suffering from fever. These seven patients did not suffer from any chronic or immunosuppressive diseases [113].

Preliminary studies indicated rodents as the most important reservoir, but even urban hedgehogs might play a crucial role in the maintenance of this pathogen, especially in human dwellings, as also confirmed by Barakova *et al*. (2017) [128, 129]. Recently, *Candidatus* N. mikurensis was also detected in dogs from Germany and Nigeria, as well as in bank voles from France and Sweden [130-133].

All the data generated in the last two decades, led to the hypothesis that *Candidatus* N. mikurensis is an emerging pathogen, which need further investigation to elucidate spreading, maintenance, and potential reservoir hosts in order to assess the risk of infection for humans and animals.

Rickettsiae

Rickettsiae are Gram-negative, obligate, aerobic, intracellular bacteria, which infect eukaryotes. In humans, they infect blood vessels (i.e., endothelial cells) and survive freely within the cytosol of the host cell.

Rickettsiae are traditionally subdivided into two groups: the typhus and the spotted fever group (SFG), which are prevalently associated with ixodid ticks. Bacterial infection in ticks is transmitted both horizontally (transstadially) and vertically (transovarially). Indeed, ticks are vectors and reservoirs of these pathogens, with vertebrates suspected to be only accidental reservoirs of rickettsiae [134].

The presence of tick-borne rickettsiae has been reported from almost all European countries, with *R. helvetica*, *R. monacensis*, *R. massiliae*, *R. felis*, *R. typhi*, *R. prowazekii*, *R. akari*, *R. conorii*, *R. slovaca*, *R. sibirica mongolotimonae*, *R. raoultii*, and *R. aeschlimanni* isolated from vectors or mammalian hosts. Some of them have been implicated in human diseases or reported as emerging pathogens, and the first three species are the most abundant in Europe [129, 134-138].

"Candidate" species have been found in ticks throughout Europe (i.e. "*Candidatus* Rickettsia kotlanii", "*Candidatus* Rickettsia barbariae," and "*Candidatus* Rickettsia vini") [139-141]. However, their pathogenic role has to be clarified, because numerous rickettsiae are regularly

reported as symbionts, microsymbionts, or endosymbionts (living in endocellular symbiosis) in ticks, as is the case of *Midichloria mitochondrii*, a bacterium belonging to the order Rickettsiales [142-144].

Prevalence rates of Rickettsiae-infected ticks are highly variable, varying from 0.5% up to 66% (*R. helvetica* in Netherlands) [145]. Higher rates are concomitant with the period of major activity of ticks (from May to September/October) [146]. Infected hosts present generic flu-like symptoms, such as fever, headache, muscle aches, cough and rash, and the severity of the infection is determined by the degree of bacteria growth.

The presence of Rickettsiae was also confirmed in *I. ricinus* ticks collected in many urban and peri-urban sites of several European countries, increasing the risk for human and animal infection [13].

***Babesia* spp.**

Babesia is a protozoan parasite, which infects and destroys red blood cells of hosts. The consequent disease, babesiosis, is an important issue in veterinary medicine worldwide, because ticks belonging to genus *Rhipicephalus* (i.e. *R. microplus* and *R. annulatus*) can transmit the parasite to cattle, which causes huge economic loss worldwide, estimated in billion dollars per year, but even to domestic animals, including horses, sheep, goats, pigs, and dogs.

In Europe, *Babesia divergens*, *B. venatorum* (EU1), and *B. microti* are the main responsible for human babesiosis, occurring prevalently in splenectomised or immunocompetent patients, and transmitted by *Ixodes ricinus* [147-150]. While most infections pass without symptoms, some people may become ill and present with flu-like symptoms such as fever, chills, muscle ache, fatigue, as well as jaundice. Severe cases, affecting the kidneys or the lungs, may occur and lead to death. However, the list of human-infecting *Babesia* species may increase, in reason of the wide spectrum of *I. ricinus* hosts, as well as the introduction of unknown wildlife protozoan species in urban and peri-urban areas.

Infection rates of *Babesia* spp. in ticks are usually rather low, ranging from 0.9 to 20%, and its transmission in tick may occur both transstadially and transovarially [151-154].

As previously mentioned, *Babesia* species are able to infect a wide range of hosts, including ungulates (roe deer, fallow deer, red deer, mouflon, and sheep), splenectomized rats, as well as non-splenectomized reindeer, sheep, and gerbil [152]. Collected data from European countries led to the conclusion that *B. divergens*, *B. venatorum*, and *B. microti* represent a potential risk in rural and in peri-urban areas [69, 129, 155-163].

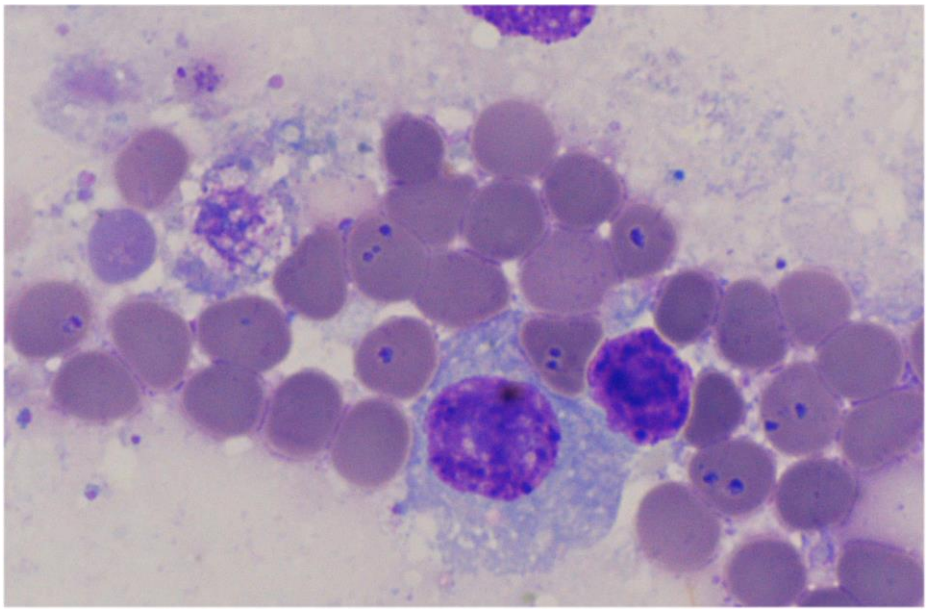


Figure 14. Peripheral blood smear with Babesia *spp.-infected erythrocytes. Magnification 1000x (oil immersion). Picture was a kind gift from Prof. Marco Genchi.*

Francisella tularensis

Francisella tularensis is the causative agent of human and animal tularemia. It is a highly infectious, Gram-negative zoonotic coccobacillus, which has been isolated from over 250 species of wild and domestic mammals, birds, reptiles, amphibians, fish and invertebrates [164]. Of the four *F. tularensis* subspecies currently known, only two are often associated with human and animal disease: the most virulent *F. tularensis* subsp. *tularensis* (type A), found only in North America, and *F. tularensis* subsp. *holarctica* (type B), present in the North America, Europe and Asia.

Francisella tularensis is important due to its easy dissemination, multiple routes of infection, and high environmental contamination. However, the most important features are the morbidity and mortality rates that it causes. For these reasons, it is classified as potential bioterrorism agent. It can be transmitted by several routes, including direct contact with infected blood and tissues through wounds, intact skin and mucous membranes, ingestion of contaminated food or water, inhalation of aerosols and arthropod bites [164].

Ixodid ticks, together with other biting arthropods, play an important role in the persistence of infections in nature and their role as potential vectors to humans and animals has been shown [165, 166]. In Europe, *I. ricinus* and *D. reticulatus* are the most responsible species in bacterium transmission to humans, who develop tularemia after tick bites in 13% to 26% of cases [167, 168]. However, prevalence of infected ticks is low, up to about 4% [169, 170].

Only transstadial transmission has been demonstrated in different hard tick species [166, 171-173]. As reported by Genchi *et al.* in 2015, there is no evidence for transovarial transmission of *F. tularensis* subsp. *holarctica* in the most abundant European tick species, *I. ricinus* and *D. reticulatus* [174].

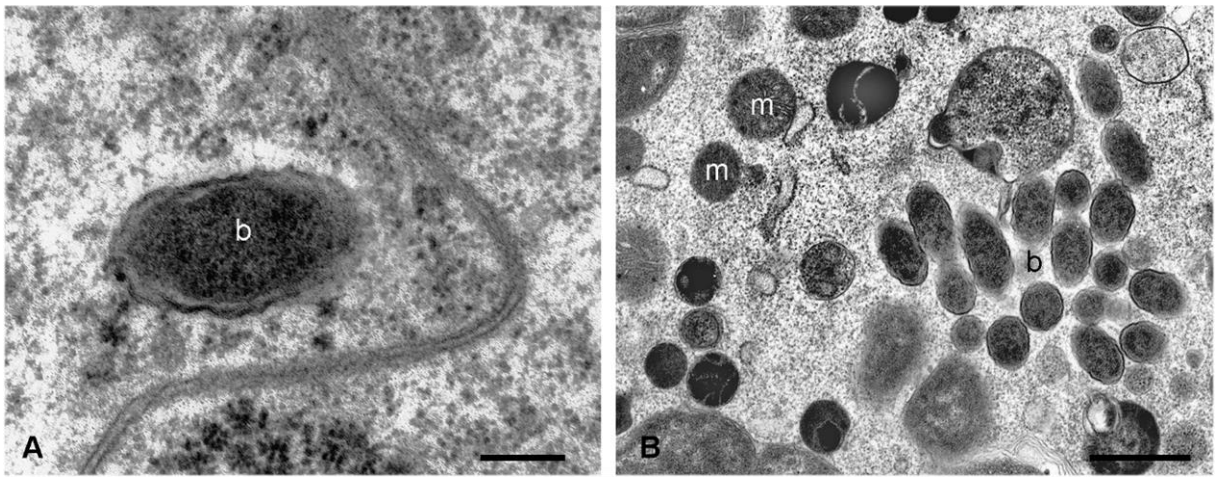


Figure 15. TEM micrographs of Ixodes ricinus *oocytes infected with* F. tularensis *subsp.* holarctica. *The morphology of some inclusions is typical of a Gram-negative bacterium, and their size is congruent with that of bacteria from the Francisella genus. Abbreviations: m (mithocondrium); b (bacterium). Scale bar: A 0.22 µm, B 1.1 µm. Adapted from Genchi* et al. *(2015) [174].*

Tick treatment and control

Currently, the treatment and control of ticks include strategies based on pharmacological and environmental treatment, as well as a behavioural education.

Several molecules have been positively proven as ectoparasiticides and can be grouped into three main categories: naturals, synthetics and new generation drugs, which is an extension of the synthetic group. However, many tick species are difficult to control, because they live in close association with vertebrate hosts or occur in areas not easily accessible for pesticide applications or for the appearance of drug-resistance strains [53].

Natural compounds

Natural ectoparasiticides are extracted from plants, in particular from their blossoms or roots. The most noted compounds are rotenone and pyrethrins. Rotenone is an insecticidal product obtained from plant roots and leguminous shrubs. It acts as inhibitor of mitochondrial respiratory enzymes. Pyrethrins are mainly six closely related insecticidal substances extracted from the pyrethrum plant, *Chrysanthemum cinerariaefolium*. They quickly penetrate the arthropod and act against the nervous system, blocking the Na^+ and K^+ transmissions [175]. Pyrethrins are important for their wide range of safety, but are rapidly biodegradable. Usually, natural ectoparasiticides are associated with other synergic molecules, which increase the drug activity more than ten to twenty times, such as piperonyl butoxide or N-Octyl bicycloheptene dicarboximide [176]. They are available in several formulations, such as shampoos, sprays, lotions and powders. However, they need to be applied regularly, as they are non-persistent.

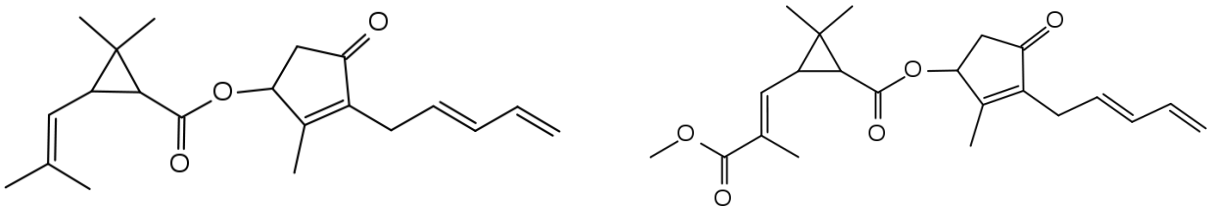


Figure 16. Chemical structure of pyrethrins. In particular of pyrethrin I (left) and pyrethrin II (right).

Synthetic drugs

Pyrethroids

The development of pyrethrins led to a new class of synthetic ectoparasiticides, known as pyrethroids. They are more stable and active than the natural molecules. They are selective for arthropods and act on their nervous system, firstly allowing excitation, followed by inhibition and paralysis [177]. Research into pyrethroid chemistry has resulted in many products, which have been classified into four generations [178]. The first includes molecules no longer in use, such as allethrin. Second generation pyrethroids include resmethrin, phenothrin and tetramethrin, while the third generation includes permethrin, which is the most commonly used pyrethroids for tick control. The last generation of pyrethroids comprises deltamethrin and flumethrin, together with cyflutrin and cypermethrin, mostly used in lice, flies and horse flies control.

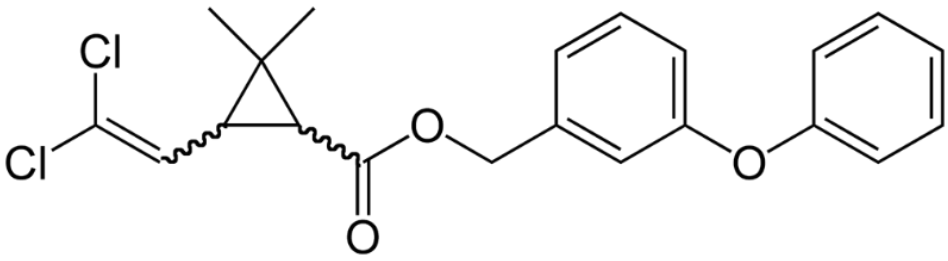


Figure 17. Chemical structure of permethrin.

As previously mentioned, permethrin acts on the voltage-sensitive sodium channels, causing neuroexcitation that leads to tick death. However, cases of resistance to permethrin have been reported in ticks of the genus *Rhipicephalus*, particularly in *R. sanguineus* and *R.(Boophilus) microplus*, probably due to a mutation in the sodium channel and/or in the esterase gene [25, 24, 26, 21].

Carbamates and organophosphates

This class of molecules acts on the nervous system, blocking the acetylcholine esterase (AChE) activity and allowing the accumulation of the acetylcoline neurotransmitter, with subsequent hyperexitation. Organophosphates result in an irreversible inactivation, while the effects of carbamates are reversible. They can cause severe neurotoxicity, as well as respiratory paralysis and death, in animals and humans. Despite this important drawback, several are still currently

used in animal products for tick control (i.e. coumaphos and diazinon). Although they belong to a different class of molecules, carbamate compounds (e.g. carbaryl and propoxur) show the same mechanism of action. However, there are no longer products available for tick control, also due to cases of resistance that have been reported over the years [16-20, 179]. Resistance mechanisms are due to mutations of the acetylcholine esterase or to the presence of a second form of the enzyme, derived from a duplication and diversification of the original one [180-183].

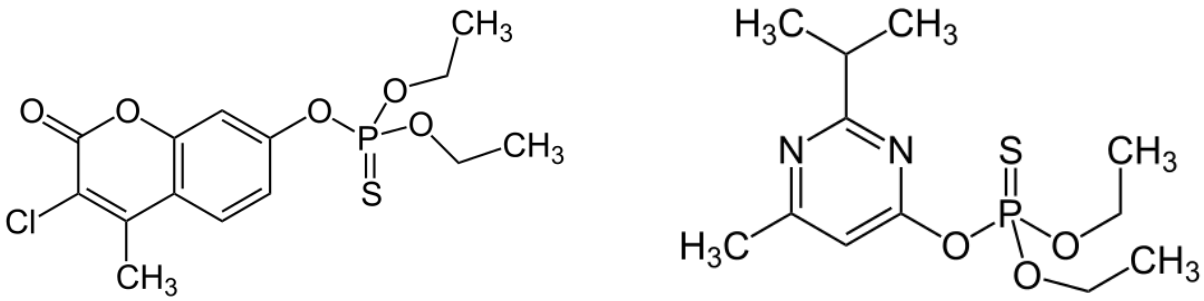


Figure 18. Chemical structure of coumaphos (left) and diazinon (right).

Formamidines

The last class of synthetic molecules, formamidine, includes another of the most used ectoparasiticide: amitraz. Its mechanism of action, although not completely understood, is completed in the nervous system. It acts as inhibitor of the MAO (MonoAmine Oxidase) enzymes and as agonist of the α- and β-adrenergin-like octopamine receptors [184]. However, amitraz show several side effects, such as transient sedation, lethargy, pruritus, bradycardia, hypothermia, hypotension, hyperglycemia and hyperexcitability (very uncommon), because of its agonist role [185, 186]. Moreover, amitraz-resistance phenomena have been reported, and they are probably due to several mutations in a putative octopamine receptor [187, 188].

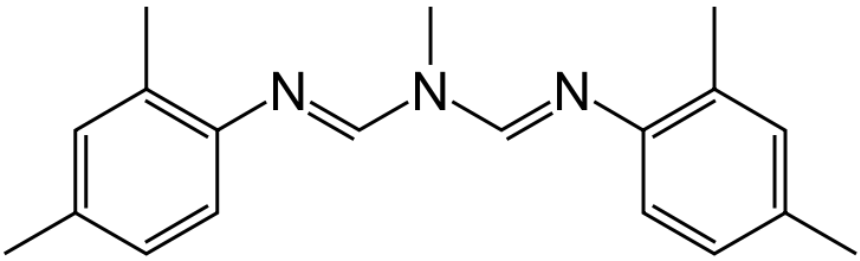


Figure 19. Chemical structure of amitraz.

New generation drugs

These antiparasitic drugs have been synthetized since the ‘90s and act through different mechanisms of action. This group comprises phenylpyrazoles, neonicotinoids, insect growth regulators, macrocyclic lactones, and the more recent isoxazolines.

Phenylpyrazoles

The most important insecticide of this class, involved in tick control, is fipronil, developed in the mid ‘90s. It is highly selective for arthropods and acts on their nervous system, blocking the GABA (γ-aminobutyric acid)-gated chloride channels [189]. Many formulations are available and clinical efficacy has been widely documented. Moreover, fipronil is combined with several other active ingredients. The first was a spot-on formulation with (S)-methoprene, now available in association with amitraz, or other pyrethroids (e.g. etofenprox or cynophenothrin) and further formulations will likely become available, as the fipronil patent expired several years ago [2]. However, as in the previous pharmacological class, recent drug-resistance cases have been reported even in phenylpyrazoles [23].

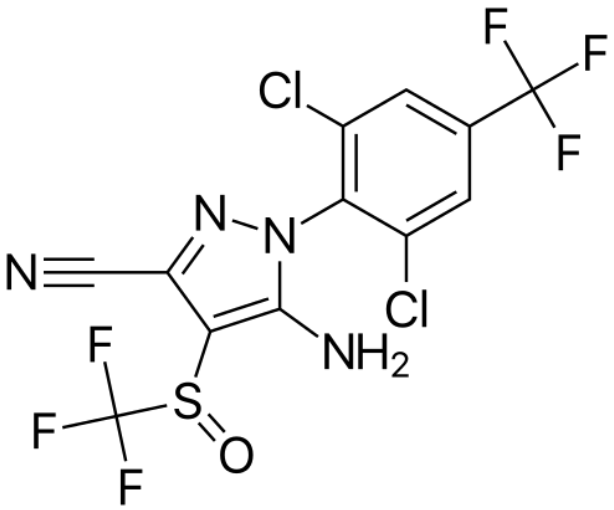


Figure 20. Chemical structure of fipronil.

Neonicotinoids

This class of drugs includes imidacloprid, introduced onto the market in 1991. It specifically inhibits the nicotinic acetylcholine receptors (nAChRs) of insects, damaging their neurological functions [190, 191]. To the same class belongs even nitempyram, prevalently used for flea and mite control [2].

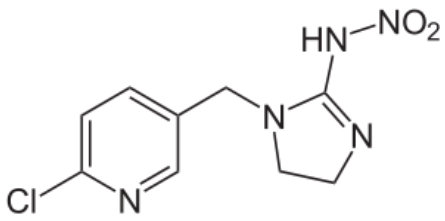


Figure 21. Chemical structure of imidacloprid.

Insect Growth Regulators

Several ectoparasiticides on market increase their strength combining these molecules with another class of new generation drugs, the IGRs (Insect Growth Regulators). They are hormone-like molecules that directly act on eggs or juvenile stages, preventing the growth toward adultness. In the last class is grouped methoprene, which is associated with natural pyrethrins and pyrethroids, and pyriproxyfen (see Table 3) [2].

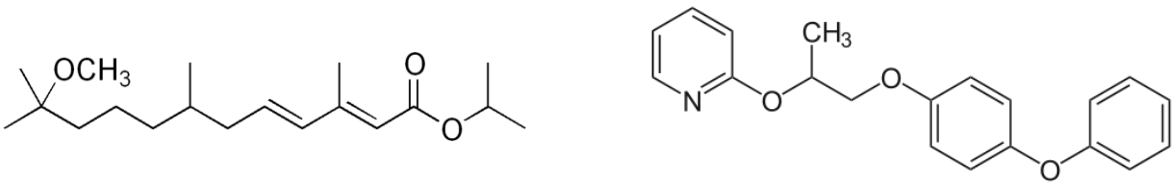


Figure 22. Chemical structure of methoprene (left) and pyriproxyfen (right).

Macrocyclic lactones

The avermectin drugs (ivermectin, abamectin, doramectin, eprinomectin and selamectin), together with milbemycins (milbemycin oxime and moxidectin) constitute the class of macrocyclic lactones (MLs).

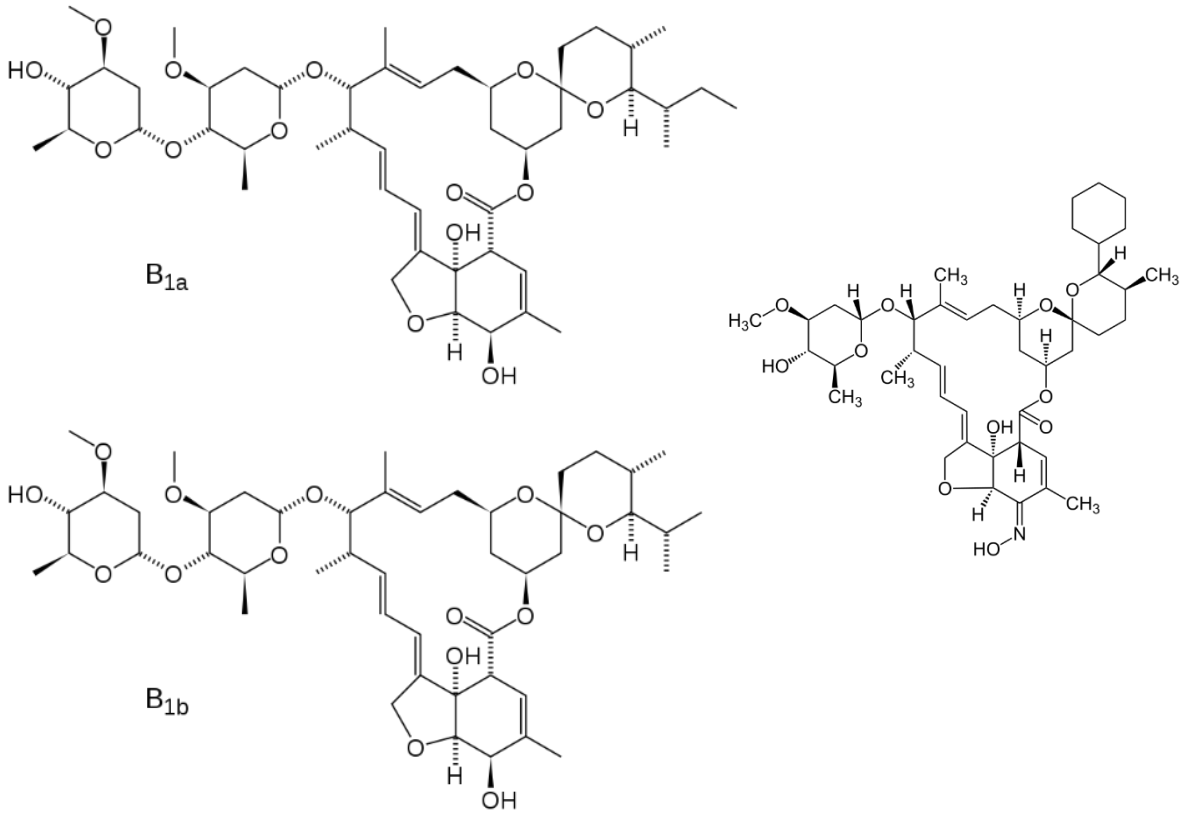


Figure 23. Chemical structure of the two isomers constituting the ivermectin (B1a and B1b, left) and of selamectin (right).

Avermectins and milbemycins have minor differences in some substituents, but they share the same general structure that confers on them the ability to bind to chloride channel receptors [192]. One main mechanism by which the MLs exert their effect is by binding ligand-gated chloride channels in the nervous system [193, 194]. Binding of glutamate-gated chloride channels, which are specific to invertebrates, causes influx of chloride ions into the parasite neurons leading to hyperpolarization, paralysis, and death [194, 195]. It also affects the reproduction of some parasites by diminishing oviposition or inducing abnormal oogenesis. Avermectins were isolated from the fermentation of the bacterium *Streptomyces avermitilis* in 1973 by Satoshi Ōmura, Nobel Prize winner, together with William Campbell, in Physiology or Medicine in 2015. Ivermectin, the first derivative, safer and more effective, was commercialised in 1981 [196]. It is constituted by two isomers, B_{1a} and B_{1b}. The other molecules derive from mutant strains of bacterium (doramectin), or from synthetic modification of natural compounds (selamectin, eprinomectin, moxidectin) [197].

Isoxazolines

Isoxazolines are the newest chemical class of pesticides, developed in the 2010s. They entered the market in 2013 as veterinary products against fleas and ticks in dogs. However, they have a broad spectrum of activity, as they are also effective against numerous agricultural and other ectoparasites. Isoxazolines block the transmission of neuronal signals in arthropods, as they are non-competitive GABA receptor antagonists and bind to chloride channels in nerves and muscle cells [198]. Affected parasites are paralysed and die. Isoxazolines approved for veterinary use have a systemic mode of action. Ingested or topically administered, isoxazolines are rapidly absorbed into blood and distributed throughout the whole body of the host. Blood-sucking parasites are killed during their blood meal [199-201]. Whether killing is fast enough to prevent disease transmission is not yet known.

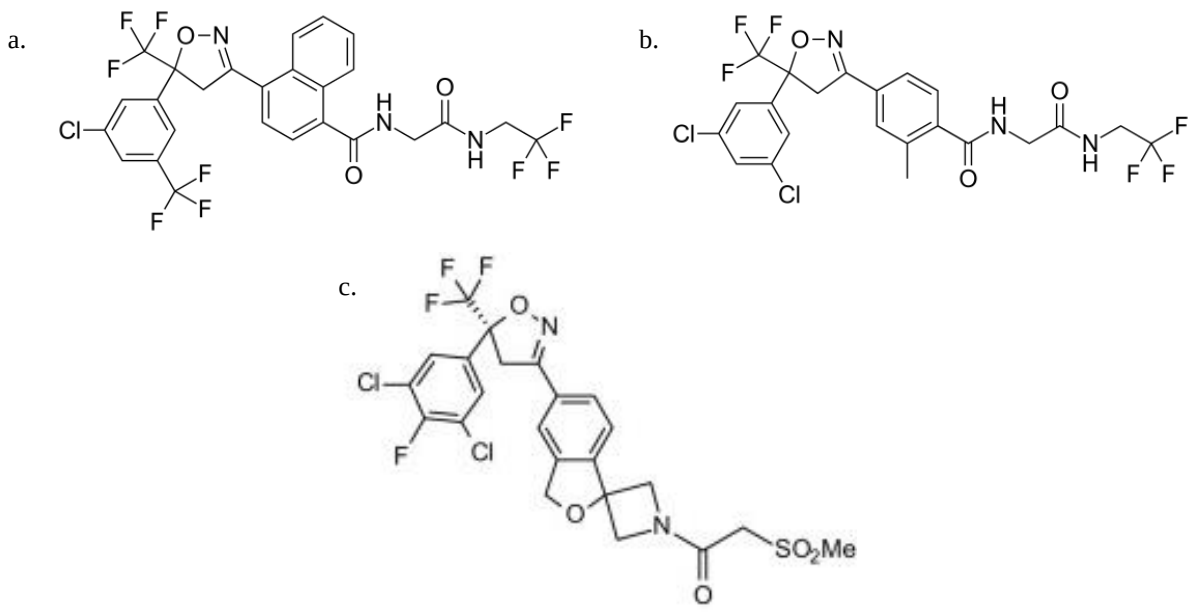


Figure 24. Chemical structure of afoxolaner (a), fluralaner (b) and sarolaner (c).

Table 3. List of the principal classes and active pharmaceutical ingredients used in tick control.

Class active pharmaceutical ingredient	**Available products**	**References**
Organophosphorus compounds		
diazinon coumaphos	collars, lotions, powders	[202]
Formamidine		
amitraz	collars, spot-on, lotions	[203-205]
Pyrethrins		
pyrethrin + piperonyl butoxide, w/ or w/o dichlorophene	solutions	
Pyrethroids (generation II)		
pyrethrin + pyriproxyfen tetrametrin even in association with: - phenothrin - phenothrin + piperonyl butoxide	shampoos, powders, sprays, solutions	
Pyrethroids (generation III)		
flumethrin, even associated with propoxur permethrin even in association with: - piperonyl butoxide + pyrethrin/tetrametrin/phenothrin - dinotefuran + pyriproxyfen	shampoos, lotions, collars, spot-on, tablets	[206-213]
Pyrethroids (generation IV)		
deltamethrin	collars	[202, 214-217]
Phenylpyrazoles		
pyriprole fipronil even in association with: - permethrin + butylated hydroxy toluene - methoprene - (S)-methoprene + eprinomectin + praziquantel	sprays, spot-on, tablets	[206, 207, 210-213, 218-222]
Neonicotinoids		
imidacloprid, even in association with: - flumethrin - permethrin - moxidectin	collars, spot-on, tablets	[212, 223, 224]
Avermectins		
ivermectin selamectin	spot-on	[202, 225-228]
Isoxazolines		
afoxolaner fluralaner sarolaner	chewable tablets	[199-201, 224-226, 229, 230]

2. Tick cell lines

History and features of tick cell lines

As previously emphasized, the need to understand tick biology and to develop effective control strategies is due to the heavy health and economic burden that TBDs represent worldwide. The increasing burden of TBDs has led to the development of new tools for understanding tick-host-pathogen interactions. Continuous cell lines are one of the most interesting developments in this light.

Even if attempts of tick cells cultivations date back over sixty years, the first results were reported only in the mid '70s. Initial attempts at establishing tick cell lines resulted in primary cultures able to survive for up to six months and were prevalently used for virus and bacteria propagation. Improvements in methodology and the expending of huge efforts over the decades led to the final purpose of generating continuous tick cell lines. The first paper describing the establishment of three cell lines from *Rhipicephalus appendiculatus* was published in 1975 by Varma *et al*. [231].

Further studies describing the establishment of cell lines deriving from other tick genera and species have been reported during the following years. In particular, they derived from *Ixodes scapularis*, *Rhipicephalus sanguineus*, *R.* (*Boophilus*) *microplus*, *Haemaphysalis spinigera*, *Hyalomma anatolicum anatolicum*, *Dermacentor variabilis* and *D. parumapertus* [232-237]. As reported by Munderloh and Kurtti, about twenty cell lines were available from eight ixodid tick species in 1995, which became more than 40 from thirteen ixodid ticks in only ten years, as reported by Bell-Sakyi *et al.* in 2007 [238, 32]. The increased number of tick cell lines was even due to improvements in medium formulations and in the use of several supplements in order to sustain cell growth [235, 239]. Despite their medical importance in birds and mammals, attention given to argasid ticks was certainly less, resulting in much slower progress in development of *in vitro* culture systems. The first continuous argasid cell line was established in about 30 years from the soft tick *Cario capensis* [240]. Currently, six more cell lines are available from the soft tick *Ornithodoros moubata* [241].

The attempts of establishing tick cell lines are time-consuming processes and the overall success rate is very low. It can take up to 5 years from the time the primary culture is initiated, and the final result is certainly conditioned by the operator experience: almost the totality of the

tick cell lines currently available have been established by T.J. Kurtti, U.G. Munderloh and L. Bell-Sakyi [32].

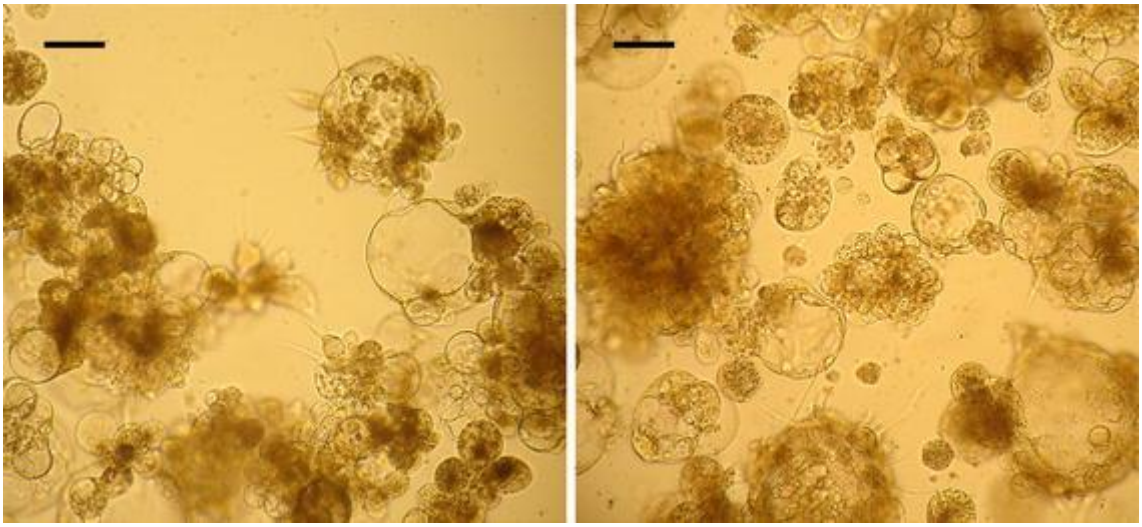


Figure 25. Ornithodoros moubata *cell lines OME/CTVM21 (*left*) and OME/CTVM22 (*right*) at passage 6, 36 months after initiation. Live, inverted microscope; scale bar = 100 µm Adapted from Bell-Sakyi* et al. *(2009) [187].*

Establishing tick cell cultures

As reported by J. Rehacek, it is convenient to divide *in vitro* tick cultures into four categories:

- haemocyte cultures;
- organ cultures;
- primary tissue and cell cultures;
- continuous cell lines [242].

Hemocyte cultures

As previously mentioned, haemocytes are specific cells present in the haemolymph that carry out important physiological functions in metabolic processes. Some types of haemocytes, for example, are involved in immunity, as they show phagocytic properties. Moreover, the major interest in this cell type lies in their role in the development and distribution of viruses and bacteria transmitted by arthropods. The preparation and maintenance of haemocyte cultures is relative easy and several of them have been developed from different ixodid and argasid tick genera (*Dermacentor*, *Hyalomma*, *Riphicephalus*, *Alveonasus*, *Ornithodoros* and *Argas*). Although their use has been strongly limited in the past, they could be a useful tool in

understanding the host-pathogen relationships and the defence mechanisms of ticks [242-244]. The main problem in haemocyte cultures is the laborious preparation, and in the number of ticks indispensable to obtain a sufficient quantity of cells, because hemocytes do not multiply regularly *in vitro*.

Organ cultures

The advantage of organ cultures lies in the preservation of the original structure, which allows the maintenance of the original properties of the host organism. Two types of organ cultures have been developed in ticks, which have been fundamental for the study of several pathogenic microorganisms.

The first is a common explantation of the organs of interest, or small pieces of them. This approach represented the first attempts to establish tick cell cultures *in vitro*, and guaranteed good viability percentages within a few days. It was prevalently used for the study of the complex life cycle of hemosporidians, *Theileria* and *Babesia*, and viruses.

The second type of organ culture is the so-called "backless tick explants", consisting in *in vitro* cultivation of ticks lacking of their dorsal integument. It is easy to perform and, although organs are not completely isolated from each other, might allow better results than in excides individual organs, as it provides maintenance conditions closely related to those in *in vivo* ticks. However, the "backless tick explant" method did not find wider application in practice. Their main application was for the study of the development of theileriae sporoblasts to sporozoites.

Excised salivary glands and the male reproductive apparatus were the principle *in vitro* organ cultures of ticks, and used for various physiological studies. Salivary glands have been used for the comprehension of the processes behind the regulation of the volume and composition of tick fluids, while the male reproductive apparatus was used for the study of the maturation process of spermatozoa. However, even organ cultures have several drawbacks, because the preparation is time-consuming and requires experienced workers.

Primary tissue cultures

Primary tissue cultures were the first results in the attempts to develop tick cell lines. However, the proliferation rate observed in the cultivation of several tissues was mostly due to migrating cells and significant for only a limited period. These cultures have been developed through two methods, which used different starting sources: embryos from eggs, developing adults within metamorphosing engorged nymphs, or whole moulting larval explants [245].

Embryonic cultures from eggs or from embryonic tissues have been generated from different tick genera and species. They grew well during the first 2 or 3 weeks, and could be split several times before they died off. Eggs were sterilised, incubated and cracked by gentle pressure with a glass rod or syringe plunger. Embryonal fragments were washed, broken by further pipetting, centrifuged and seeded into sterile flasks. The presence of egg shells was not important for the maintenance of the cultures and removed through subsequent washes. The main problem of this approach was represented by the frequent bacterial and fungal contaminations. A possible solution to this issue was the use of eggs deposited in aseptic environments by surface-sterilised engorged females, using a solution of 70% ethanol or 10% benzalkonium chloride followed by washes in sterile distilled water.

On the other hand, preparation of primary tick cultures from tissues of adults from nymphs undergoing metamorphosis was more time-consuming. Nymphs are the best source of a high number of growing cells. Ticks were dissected in sterile saline, and Malpighian tubes and the digestive tract removed. Remaining tissues were pooled, and then dissociated by enzymatic treatment (which had detrimental effect on cell growth), or the simple use of sterile scissors and forceps [231]. Another cell source for tissue cultures were ovarial tissues from partially engorged females. Fibroblast-like cells migrate during the first days of cultivation, followed by the production of small discrete colonies of epithelial-type cells in 7 to 10 days, which remained viable for few weeks, and could be sub-cultured 5 to 9 times before their death.

The success rate of the method depended on the tick species, its developmental stage and, obviously, on the chosen growth medium. Different culture media have been employed, as well as numerous supplements, which increased culture's survival for several months.

Their main application was the propagation of viruses, like the tick-borne encephalitis virus, the cultivation of rickettsiae, and the study of other biological problems. However, the preparation of a sufficient number of primary cultures for experimental studies is too laborious and time-consuming, requiring basics in tick handling and experts in the dissection techniques. These important drawbacks are the main obstacles for their broader application.

Continuous cell lines

Continuous cell lines represent one of the most important tools for researchers because they show different advantages compared to organ and primary cultures from which they derive. Firstly, they are easy to handle, and grow in relatively standardised conditions. However, not every line is characterised by a uniform cellular type, because no attention has been paid to the selection of particular tissue types [236, 237, 246].

The principle sources of cells were the same as previously described, but eggs laid by engorged females were probably the most important. Therefore, the use of ethanol solutions, as well as distilled water and antibiotics was fundamental. However, despite careful processing, employed methodologies did not protect cell cultures from internal contamination by microorganisms living in the digestive tract and haemolymph of ticks, but, fortunately, these infections were infrequent.

The most decisive step in the establishing of tick cell lines was the choice of a suitable medium, which was often represented by basic media, such as Eagle's, Hanks and Leibovitz L-15. Certainly, several adjustments were required, such as the use of supplementary aminoacids and sugars, or the control of the final pH value. Even the plastic material used for the maintenance and the development of the cell cultures could be toxic and the introduction of precautions were fundamental. As reported by J. Rehacek (1994), important measures included i) the pretreatment of culture vessels by the cultivating media, ii) the enrichment and use of conditioned media, iii) the cultivation of primary cultures for several months before attempting further passages, iv) the reseeding of cells into the same flasks (apparently accelerates the rate of cell attachment and proliferation, and v) the releasing of cells from their congested loci from the surface of culture vessels by scraping or pipetting [242].

Many other factors were crucial for the development of continuous tick cell lines. The first was represented by the tick genus and species. As mentioned previously, the first three established cell lines derived from ticks of the species *R. appendiculatus* in 1975, and all efforts to obtain cell lines from the genus *Ixodes* did not give positive results, leading to the wrong consideration that some genera of ticks were not suitable for the establishment of cell lines. To date, in fact, at least eleven embryonic cell lines from the genus *Ixodes* are available (Table 4).

Table 4. List of tick cell lines available in 2007, as reported by Bell-Sakyi et al. *(2007) [32]*

Tick species	Instar	Cell lines
Amblyomma americanum	Embryo	AAE2, 12
Amblyomma variegatum	Larva	AVL/CTVM13, 17
Rhipicephalus (*Boophilus*) *decoloratus*	Embryo	BDE/CTVM16
Rhipicephalus (*Boophilus*) *microplus*	Embryo	*B. microplus* IX, VII-SCC
		BME26
		BME/CTVM2, 4, 5, 6
Carios capensis	Embryo	CCE1, 2, 3, 5
Dermacentor albipictus	Embryo	DALBE3
Dermacentor andersoni	Embryo	DAE3, 15, 100
Dermacentor (*Anocentor*) *nitens*	Embryo	ANE58
Dermacentor variabilis	Embryo	DVE1
Hyalomma anatolicum anatolicum	Embryo	HAE/CTVM7, 8, 9, 10, 11
Ixodes scapularis	Embryo	IDE2, 8, 12
		ISE5, 6, 18, 25
Ixodes ricinus	Embryo	IRE11
		IRE/CTVM18, 19, 20
Rhipicephalus appendiculatus	Embryo	RAE25
		RAE/CTVM1
	Nymph	RA243, 257
		RAN/CTVM3
Rhipicephalus sanguineus	Embryo	RSE8

Characteristics of tick cell lines

Compared to *in vivo* ticks, continuous cell lines show an important difference in genomic assessment, because they are characterised by the presence of aberrant euploid cells (i.e. cells with gained and/or lost chromosomes). However, this does not appear to influence their survival rate, as reported by Chen *et al.* in 1994 [247].

Besides this exception, tick cell lines share common characteristics with the arthropods from which they were derived, especially the growth environment. As ticks are hematophagous arthropods feeding on a wide range of hosts, cell cultures tolerate extremely well mammalian culture media supplemented with mammalian serum. The incubation temperatures are similar to those in which ticks live, ranging between 28 °C and 34 °C, but some lines are adapted to

higher temperatures (i.e. 37 °C). pH values are also important, because many cell lines prefer environments similar to those in developing larvae, nymphs and adults (with pH ranging from 6.5 to 6.8), while others grow in neutral or alkaline environments, enabling growth of acid-sensitive pathogens [238, 248].

Cultured tick cells require high cell densities (up to 10^6-10^7 cells/ml). They are generally not strongly adherent, and do not exhibit contact inhibition. Thus, they grow as a combination of three-dimensional incomplete cellular layers and suspension cultures. Continuous cell lines show a relatively slow proliferation rate, and many of them do not require regular subculture. The last feature makes cell cultures particularly useful for isolation of slow-growing microorganisms. Moreover, individual cultures can be preserved for long periods of time, even several years, with only regular medium changes and occasional subcultures, reflecting the important ability of ixodid ticks in nature to face long intervals between blood meals [32].

Continuous tick lines can be cryopreserved in liquid nitrogen for many years, but frozen cells are difficult to resuscitate reliably. In compensation, they support temperatures of 12 °C or even 4 °C well, which are preferable for short-term storage [246, 249, 250].

It is crucial to underline the main feature of tick cell lines: they are generally composed of two or more cell types and the proportion between them varies within a single culture and at different passage levels [251]. Attempts to clone single cell types have been carried out, but no positive results have been achieved [237]. Consequently, the mixture of cells seems to be essential for the survival of the entire culture.

Applications of tick cell cultures

Since their origin, *in vitro* cell cultures have helped researchers in solving several issues. The use of the first three tick cell lines from *R. appendiculatus* were used for the isolation, propagation, and study of arboviruses (arthropod-borne viruses). Other applications of tick cultures studied the *in vitro* development of tick-borne protozoa and bacteria, which in the last decades took advantage of the great improvements in molecular genomics and proteomics.

Tick cell cultures in virology

Tick cell cultures have been used for the study of pathogenesis and evolution of viruses, in particular of arboviruses, but also of Langat, Quaranfil (Quaranjavirus genus), West Nile and Louping-ill (Flaviviridae family) viruses. Some viruses can establish chronic infections without visible inclusions or cytopathic effects on the cultures. It is remarkable that arboviruses

infections can be maintained indefinitely for the entire period of the subculture, while in mammalian hosts, both *in vivo* and *in vitro,* they result in death. The precise molecular basis of this difference is not completely known, but preliminary studies with immunoelectron microscopy demonstrated differences in the site of appearance of the individual structural virus proteins, and in their pattern of movements and dispersal through the infected cells, leaving tick cells intact and disrupting mammalian ones [252]. Tick cell lines can be used to isolate many other viruses, such as wild-type tick-borne viruses from field material and mosquito-borne viruses (e.g. Chikungunya, O'nyong, yellow fever, St. Louis encephalitis, and Semliki Forest virus) [234, 253]. In fact, tick cell cultures are able to support more than 60% of the mosquito-borne viruses tested, whereas few tick-borne viruses grow in mosquito cells [242]. However, tick cell cultures can harbour "natural" pathogens, as in the embryo-derived cell lines from *I. scapularis*, IDE2 and IDE8, which were found to be chronically infected by the St Croix River virus. It is an orbivirus transmitted transovarially (vertically, from one generation to the next through the eggs), which is absent from the other cell line ISE6 [32].

Tick cell cultures in microbiology

Tick cell cultures have been extensively used in cultivation of obligated intracellular bacteria. The range of these prokaryotes has been extended and includes several bacterial pathogens of considerable medical and veterinary importance worldwide. The main studied agents belong to the family Anaplasmataceae, above all from genera *Ehrlichia* and *Anaplasma*, but even to the family Rickettsiaceae [254]. Cell lines derived from various tick species, in particular *I. scapularis*, but also *I. ricinus*, have been used for the continuous cultivation of these pathogens [237, 32]. Interestingly, some bacterial variants grown in tick cell cultures remained infective for their respective mammalian hosts, and others, i.e. *A. marginale* and *A. phagocytophilum*, became able to infect mammalian endothelial cells *in vitro*, naturally not invaded by those pathogens. Other uses of tick cell culture concern cultivation of other bacteria belonging to the genera *Spiroplasma* and *Borrelia*. Unfortunately, to date is not possible to cultivate the emerging Anaplasmataceae bacterium *Candidatus* Neoehrlichia mikurensis [13].

However, field ticks can harbour bacterial endosymbionts. Some of them can interfere with several physiological processes and the transmission of other pathogens, while others seem to be not harmful to humans, animals, or the ticks themselves. Sometimes they are closely related to known pathogens and their *in vitro* cultivation can help with the investigation and characterization of relationships with host cells. These endosymbionts can be maintained in the deriving cell cultures, or lost. This is the case of *Midichloria mitochondrii*, a bacterium

belonging to the order Rickettsiales, which has a prevalence of 100% in adult females, eggs and immature stages of *I. ricinus* [142, 143]. It is probably transmitted through a vertical route and the host reproductive organs are the preferred sites for its multiplication [255]. The maintenance conditions of tick cell cultures (i.e. the use of antibiotics and the constant incubation temperatures) could have a negative effect on the bacterium survival, as reported by Najm *et al.* in 2012, concerning eight embryonic cell lines deriving from four species of ticks from the genera *Ixodes* and *Rhipicephalus* [256]. The same factors might act in ticks maintained *ex vivo*, which feed on antibiotics-treated mammalian hosts [256].

Tick cell cultures in parasitology

Organ, primary and continuous cell lines have been used for isolation and propagation even of protozoa and other parasites. However, it seems that cell lines may differ in the ability to support babesial vector stages, independently of the species of origin of the line, although the infection of a *R.* (*Boophilus*) *microplus* cell line with *Babesia bovis* resulted in the appearance of sexual stages, both intra- and extra-cellularly. This is in contrast to organ cultures, leading to presume that tick cell lines do not provide a convenient substrate for their cultivation.

Moreover, tick cell lines from the genus *Rhipicephalus* can be infected by promastigotes of *Leishmania* species, in particular of *Leishmania major* and *L. donovani*. As in sandfly (in particular *Phlebotomus*) and mammalian hosts, flagellated promastigotes became non-motile intracytoplasmatic amastigotes in tick cultures.

Even Apicomplexa, as the cyst-forming coccidian *Besnoitia besnoitii* (Sarcocystidae, Toxoplasmatinae) that causes besnoitiosis in cattle, and microfilariae of the genus *Oncocerca* can be cultivated in tick cell cultures.

The last important application of cultured cell lines is the study of the drug resistance mechanisms in ticks. According to this topic, cell cultures offer the most attractive alternative in establishing of *in vitro* models, which might replace the *in vivo* approach, based on tick populations in which acaricide resistance has already been established in the field. The interest in tick cell lines depends on several advantages, ethical *in primis*, but also practical, such as the easily controlled experimental parameters, the reduced variability among experiments and the cost-effective approach. Two papers described the establishment of cell lines from *R.* (*Boophilus*) *microplus* resistant to acaricides. Cossio-Bayugar *et al.* in 2002 reported the generation of a cell line resistant to the organophosphate coumaphos, while Pohl *et al.* in 2014 to ivermectin. Interestingly, Pohl *et al.* were able to draw attention to the role of ABC transporters on the onset of resistance mechanisms in ticks [257, 34].

Table 5. Microorganisms propagated in tick cell lines since 1995. Adapted from Bell-Sakyi et al. *(2007) [32].*

Microorganism species	**Mammalian host (disease caused)**	**Tick cell line(s) used**
Anaplasma marginale	Cattle (anaplasmosis)	IDE8, ISE6, IRE/CTVM18
Anaplasma phagocytophilum	Domestic ruminants (tick-borne fever), horses (equine granulocytic ehrlichiosis), humans (human granulocytic anaplasmosis)	IDE8, ISE6
Anaplasma ovis	Sheep (anaplasmosis)	IDE8
Anaplasma sp.	White-tailed deer	ISE6
Anaplasma sp. (Omatjenne)	Cattle	IDE8
Borrelia burgdorferi	Rodents, dogs, humans (Lyme disease)	ISE6, IDE8
Borrelia lonestari	Humans (southern tick-associated rush illness)	ISE6
Ehrlichia canis	Dogs (canine ehrlichiosis/tropical canine pancytopaenia)	IDE8, ISE6, IRE/CTVM18
Ehrlichia chaffeensis	Humans (human monocytic ehrlichiosis)	ISE6
Ehrlichia ruminantium	Cattle, sheep, goats,wild ruminants (heartwater, cowdriosis)	AVL/CTVM13, 17, BDE/CTVM16, BME/CTVM2, 6, IDE8, IRE/CTVM18, RAE25, RAE/CTVM1, RAN/CTVM3
Rickettsia rickettsii	Humans (Rocky Mountain spotted fever)	IDE2, DALBE3, ISE6, IDE8
Rickettsia peacockii		DAE100, ISE6, BME26, DVE1, DAE3, DAE15, IDE12, IDE2, IDE8, IRE11, CCE3
Rickettsia monacensis	?	ISE6, IRE11, DAE100, IDE8
Rickettsia helvetica	Humans (fever, perimyovarditis)	IRE11

continued

Microorganism species	Mammalian host (diseased caused)	Tick cell line(s) used
Rickettsia montanensis	Various small mammals	IDE2, DALBE3
Rickettsia sp. (spotted fever group)	?	RAE25, IDE2, IDE8
Rickettsia felis	Humans (flea-associated spotted fever)	ISE6
Wolbachia persica		DALBE3
Tick-borne encephalitis virus	Humans (tick-borne encephalitis)	RA257, ISE6, RAE/CTVM1, AVL/CTVM17, IRE/CTVM18
West Nile virus	Horses, humans (West Nile fever)	ISE6, RAE/CTVM1, AVL/CTVM17, IRE/CTVM18
Powassan virus	Humans (Powassan fever/encephalitis)	ISE6, RAE/CTVM1, AVL/CTVM17, IRE/CTVM18
Langat virus	Rodents, experimentally infected humans (Langat encephalitis)	ISE6, RAE/CTVM1, AVL/CTVM17, IRE/CTVM18
Louping ill virus	Sheep, other domestic/companion animals, grouse (Louping ill encephalitis)	ISE6, RAE/CTVM1, AVL/CTVM17, IRE/CTVM18
Venezuelan equine encephalitis virus	Horses, humans (encephalitis)	RAE/CTVM1
Dugbe virus	Humans (fever), domestic ruminants	ISE6
Hazara virus	Humans (no disease but related to Crimean-Congo haemorragic fever virus)	ISE6
Thogoto virus	Humans (encephalitis), domestic ruminants	RAE/CTVM1, BME/CTVM6, HAE/CTVM9

3. Drug resistance and ABC transporters

Drug resistance mechanisms

Technological advancements during the last decades have allowed the detection of the emergence of infectious diseases, which threated humans and animals. The high mortality and morbidity rates have driven the scientific community to investigate the molecular mechanisms of pathogenesis, the host-pathogen interactions, and their epidemiology, in order to develop effective measures for host survival and safety. Most of the strategies used are based on chemical drugs, which include antibiotics, herbicides, and therapeutic agents against virus, parasites, cancer, etc. Drugs usually induce chemical stress in pathogens, such as the synthesis of reactive oxygen and reactive nitrogen species [258-260].

However, the exposition to chemicals induces susceptible pathogens to evolve new processes for surviving, selected further by continuous, intermittent or fluctuating treatments. Some of these generic defences, alone or in combination, have led to resistance to many chemicals.

Among the mechanisms developed by pathogens, the most important can be related to the reduction of the probability of drug-target interactions, through decreased drug-binding affinity or to an overexpression of the target. In some cases, pathogens can inhibit an entire metabolic pathway, replacing it with the induction of an alternative pathway, or developing new strategies in order to obtain the essential metabolite from the host. Another mechanism that contributes to the development of resistance is the up-regulation of repairing pathways, as drugs act on DNA, proteins, and lipids inducing important alterations [27, 28].

The molecular mechanisms of resistance can be included in two categories, intrinsic (or natural) and acquired, according to the mode of tolerance.

Acquired mechanisms are typical of bacteria, and involve methods of gene transfer/exchange through processes of transformation, transduction, or conjugation. The latter is the most general mode of drug resistance transmission, in which exchange of genetic material (e.g. plasmids) is facilitated by a temporary “pilus” between two adjacent bacteria. Transformation is the process of uptake of exogenous DNA (e.g. deriving from lysis of other bacteria in the environment) and further incorporation into specific genetic cassette. While transduction is a vector-based

introduction of drug resistance genes into bacterial host, essentially mediated by viruses [27, 28].

However, the development of drug resistance in eukaryotic pathogens, such as parasites or fungi, is more complex and based on intrinsic mechanisms.

Intrinsic resistance

Intrinsic resistance mechanisms to antimicrobial or chemical compounds are associated with particular features of the pathogen, selected by evolution. Intrinsic resistance can be considered as an "insensitivity" to that particular compound, and may be due to several mechanisms, as reported by Gunjan *et al*. (2017), Feyereisen *et al*. (2015), and Boumendjel *et al*. (2009) [27, 28, 261].

Absence or modification of target site

In pathogens, the drug uptake is fundamental for chemical action against targets. However, some of them are able to develop particular abilities, such as the manipulation of the cell wall composition in bacteria, which alters levels of target expression (e.g. down-regulation of the membrane porin expression). In other pathogens, indeed, therapeutic pressures might select casual mutations in drug target sites. The result is the preservation of the pathogen integrity and survival. Numerous studies confirming these mechanisms have been reported in literature. A clear example is the mutation of the β-adrenergic octopamine receptor in *Rhipicephalus microplus* ticks (RmβAOR), which is associated to amitraz resistance [22].

Species-specific structure of target site

The mechanism of action of chemical compounds is similar in related pathogens, such as pathogens belonging to the same genus or family. However, it is not unusual to detect mutations of target sites among pathogen species of the same genus. Alterations of target sites lead to a fall in drug affinity and efficacy.

Another possible drug resistance mechanism involving target site is the expression of different targets with changes in structural motifs, in response to specific drugs.

Inactivation of antimicrobial agents via modification or degradation

One of the most effective mechanism of resistance is the inactivation of chemical compounds. The destruction, or manipulation of the active component is catalysed by different enzymes. A typical example of this intrinsic resistance is represented by the bacterial enzyme beta-lactamase, or other acetylases, adenylases, and phosphorylases, which are also present in higher eukaryotes. As reported by Xu *et al.* (2005), xenobiotics induced metabolising proteins of phase I and II, as well as other proteins of phase III, involved in their transport [262]. An important role is played by reductases, which are included in complex haemoproteins, cytochromes P450 (CYPs). They are present in all living kingdoms and the most important in metabolism of a plethora of drugs is the CYP3A4 [27].

Moreover, they are often associated with other enzymes involved in drug modification and inactivation, such as transferases. Duscher *et al.* in 2014 reported the implication of the enzyme glutathione S-transferase in the detoxification mechanisms against permethrin in the brown dog tick, *Rhipicephalus sanguineus*. [263].

Presence of efflux pumps

The exposure of a pathogen to high concentrations of drugs may induce the expression of particular transporters. They bind drugs in the inner side of the membrane and release them in the outer one. Some transporters are specific for a few molecules, while others are able to recognise a wide range of structurally and functionally different drugs. In prokaryotes they are divided into five families: the resistance-nodulation-cell division (RND), the major facilitator (MF), the staphylococcal/small multidrug resistance (SMR), the ATP-Binding Cassette (ABC), and the multidrug and toxic compound extrusion (MATE). All families, except ABC, are secondary transporters, which are activated by proton and sodium gradients. ABC family is composed by active transporters, which are able to hydrolyse ATP to drive efflux. ABC transporters are the main proteins involved in drug exclusion in eukaryotes (see below).

High detoxification capacity

Many pathogens, in particular bacteria and fungi, but also higher organisms (e.g. insects and other arthropods), are able to produce and secrete toxic compounds, usually for protection from external toxins. To protect themselves from the same toxic effect, they synthetize many enzymes and other molecules that are able to recognise and neutralise them, or other xenobiotics, such as drugs. In higher organisms, specific tissues are specialised in xenobiotic metabolism and detoxification processes.

Low drug delivery

Many pathogens have developed alternative detoxification processes, in order to reduce the bioavailability and stability of drugs. They are often associated with increased metabolism rate. The result is a reduced circulation time of the drug inside the host system, which contribute to low drug delivery into the target site.

Cell cycle effects

The alteration of cell division rate is one of the major causes of intrinsic drug resistance in pathogens. It is more evident in mammalian cells, where some cases of drug resistance in cancer patients are due to slow growing tumours.

Chemically-induced adaptive change

Pathogen cells respond to chemical compounds through biochemical alterations, in order to restore physiological conditions. Differently from forms of acquired resistance, adaptive changing are reversible in the absence of the drug.

Stress response

Environmental factors can induce pathogens to stimulate resistance mechanisms, which may help them in response to drugs. Examples of stress stimuli are pH, osmotic shock, UV irradiation, heat, trauma, viral infections, anoxia, and oxidative stress. Pathogen response to stressing factors includes the induction of many enzymes involved in DNA repair and cell replication (e.g. topoisomerases), as well as in apoptosis.

In conclusion, drug detoxification in eukaryotic cells is generally described as a process that involves three main steps: the chemical modification of xenobiotics, followed by conjugation to anionic groups, such as glutathione, glucuronate or sulfate, and excretion through ABC transporters [264].

Resistance to insecticides has been shown to involve enzymes from the first two classes, including esterases, CYPs and glutathione-S-transferases [265-267], but the role of ABC transporters in the onset of resistance phenomena has been recognised and deeply studied only in the last few years [268-270].

ABC transporters

The importance of transport-related proteins in cell biology is underlined by the fact that they are encoded by 10% of the *Escherichia coli* genome and the 4% of the human genome [271, 272] and the ATP-Binding Cassette (ABC) transporters are one of the largest family in all kingdoms of life. These are active membrane proteins, which need to bind and/or hydrolyse a pair of ATP molecules and use the binding and/or hydrolysis energy to either efflux specific compounds across the membrane or to flip them from the inner to the outer leaf of the membranes, against concentration gradient [272-274]. They have been conserved across the three kingdoms of archaea, eubacteria and eukarya, and are involved in a multiplicity of cellular processes such as maintenance of osmotic homeostasis, transport of metals, ions and peptides, antigen processing, cell division, bacterial immunity, pathogenesis and sporulation, and cholesterol and lipid trafficking. ABC transporters can be grouped into exporters and importers with the last group further divided into two classes (I and II), depending on details of their architecture and mechanism [275-277].

The complete transporter protein contains two transmembrane (TM) domains with of 6–11 membrane spanning α-helices, and two ATP-binding sites, also known as nucleotide binding domains (NBDs). These transporters are so called in reason of the ATP-binding subunits that are disposed as two "cassette-like" pockets. Sequence conservation can be high between the two transmembrane domains in eukaryotic transporters and close to the 30% in some proteins, suggesting that the two TM domains in these transporters are a result of gene duplication originating from homodimeric ancestors [278].

The classification of ABC transporters is based on the sequence analysis of the amino acids in the NBDs [279]. The NBDs contain sequences originally recognised in other ATP-binding proteins, and two more conserved motifs, named Walker A and B. Furthermore, ABC proteins contain additional elements, such as the "LSGGQ" signature (C) motif, found just upstream of the Walker B domain, the Q-loop, and the H-motif [272, 276, 280, 281]. ABC transporters are divided into full transporters, in which the four domains are present in one unique protein, and half transporters, in which there are only a single TM and NBD domain. As half transporters are not able to execute their function, they need to form either homo- or heterodimers to compose a functional carrier. As previously mentioned, their functions are different among prokaryotes, where ABC proteins are involved in the in-take processes of several substances,

vs. eukaryotes, where they are mainly responsible for the efflux of compounds outside the cell or in specific organelles.

Thanks to its high level of similarity among kingdoms, the prokaryotic and eukaryotic ABC transporters have been clustered into one protein family [272]. The presence of several conserved motifs and the nucleotide binding domains, is the hallmark of the ABC transporter family. Sequence identity for the NBDs within and between bacterial and eukaryotic exporters is high, with values between 30 and 50%, pointing to a similar three-dimensional fold and a conserved mechanism of energy coupling. The conserved nature of the tertiary structure can be seen in X-ray crystal structures of isolated NBDs and NBDs that are part of intact transporters [278].

Various mechanistic models have been proposed, some being contradictory. Only in the last few years, thanks to the improvement in the high-resolution study of structures, a more reliable mechanism for ABC importers and exporters has been described (Figure 26). In this model, transport substrate has to interact at one point or another with residues of the transmembrane α-helices that line the transmembrane pore. This binding allows a conformational shift in the transporter structure, which has greater affinity to ATP molecules. Subsequent hydrolysis of the nucleotides offers the energy for conformational changes that allows active transport [282, 283]. For example, over-expression of ABC transporters, which has been observed in resistant organisms, protects cells from the entry of macrocyclic lactones in helminths, limiting their efficacy. With the use of inhibitors, ABC transporters function is reduced and drugs accumulate in the cells [284].

Fifty-eight members of the ABC family have been extensively studied, including 49 human ABC genes and 9 additional genes found in other animal species. From the 58 genes, 68% are present in all vertebrate genomes. Focusing on the sequence similarity of the NBDs, the ABC protein family is divided into eight subfamilies, named from A to H. The last subfamily, ABCH, has been found only in arthropods and in zebrafish genomes, but its function is not yet determined [285, 286].

*Table 6. List of ABC transporters recognised in human and zebrafish (*Danio rerio*). Although the zebrafish genome is incompletely assembled and annotated (*), the number of members is similar. Adapted from Dean and Annilo (2005), as reported by Ferreira* et al. *(2014) [279, 287].*

Subfamily	Members	Functions	Human	Zebrafish*
ABCA	*ABCA1 to ABCA13*	Cholesterol efflux, phosphatidil choline efflux, N-retinylidiene-PE efflux	12 members	7 members
ABCB	*ABCB1 to ABCB11*	Peptide transport, iron transport, Fe/S cluster transport, bile salt transport, xenobiotics transport	11 members	9 members
ABCC	*ABCC1 to ABCC13*	Organic anion efflux, nucleoside transport, chloride ion channel, sulfonylurea receptor, potassium channel regulation, xenobiotics transport	13 members	11 members
ABCD	*ABCD1 to ABCD4*	Very long chain fatty acids transport regulation	4 members	4 members
ABCE	*ABCE1*	Elongation factor complex	1 member	1 member
ABCF	*ABCF1 to ABCF3*	Unknown function	3 members	3 members
ABCG	*ABCG1 to ABCG5*	Cholesterol transport, sterol transport, toxin transport	5 members	5 members
ABCH	*ABCH1*	Unknown function	No members	1 member

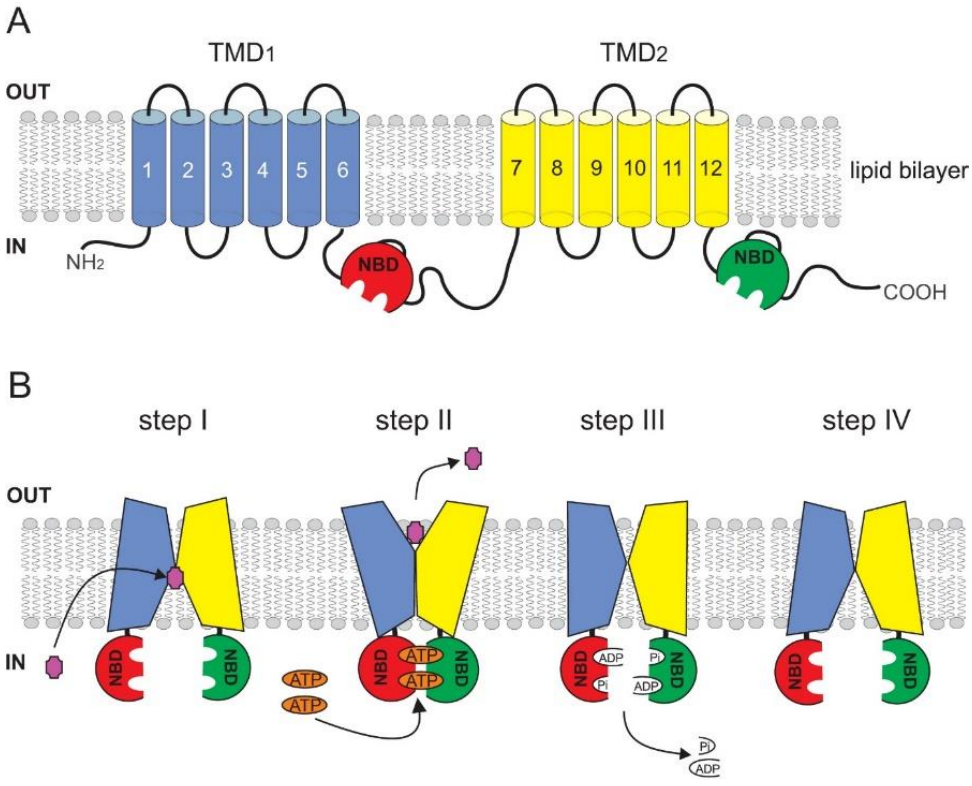


Figure 26. ABC full transporter structure and the ATP-switch model for the transport cycle of an ABC transporter (exporter-type) A) Typical structure of an ABC full transporter containing two TMDs, TMD_1 (blue) and TMD_2 (yellow) each containing 6 transmembrane (TM) segments, and two NBDs, NBD_1 (red) and NBD_2 (green). ABC half transporters have only one TMD and one NBD and need to form homo- or heterodimers to constitute a functional transporter. B) The ATP-switch mechanism. The transport cycle is enhanced by binding of a substrate (purple cross) to a high-affinity pocket formed by the TMDs (blue and yellow pentagon). Subsequently, a conformational change is transmitted to the NBDs (green and red), facilitating ATP (orange oval) binding and closed NBD-dimer formation. The closed NBD dimer induces on its turn a major conformational change in the TMDs, with TMDs rotating and opening toward the outside, initiating substrate translocation (step II). ATP hydrolysis initiates dissolution of the closed NBD dimer, resulting in further conformational changes in the TMDs (step III). Finally, phosphate and ADP release restores the transporter to the open NBD-dimer conformation (step IV). Adapted from Dermauw et al. *(2014) [268].*

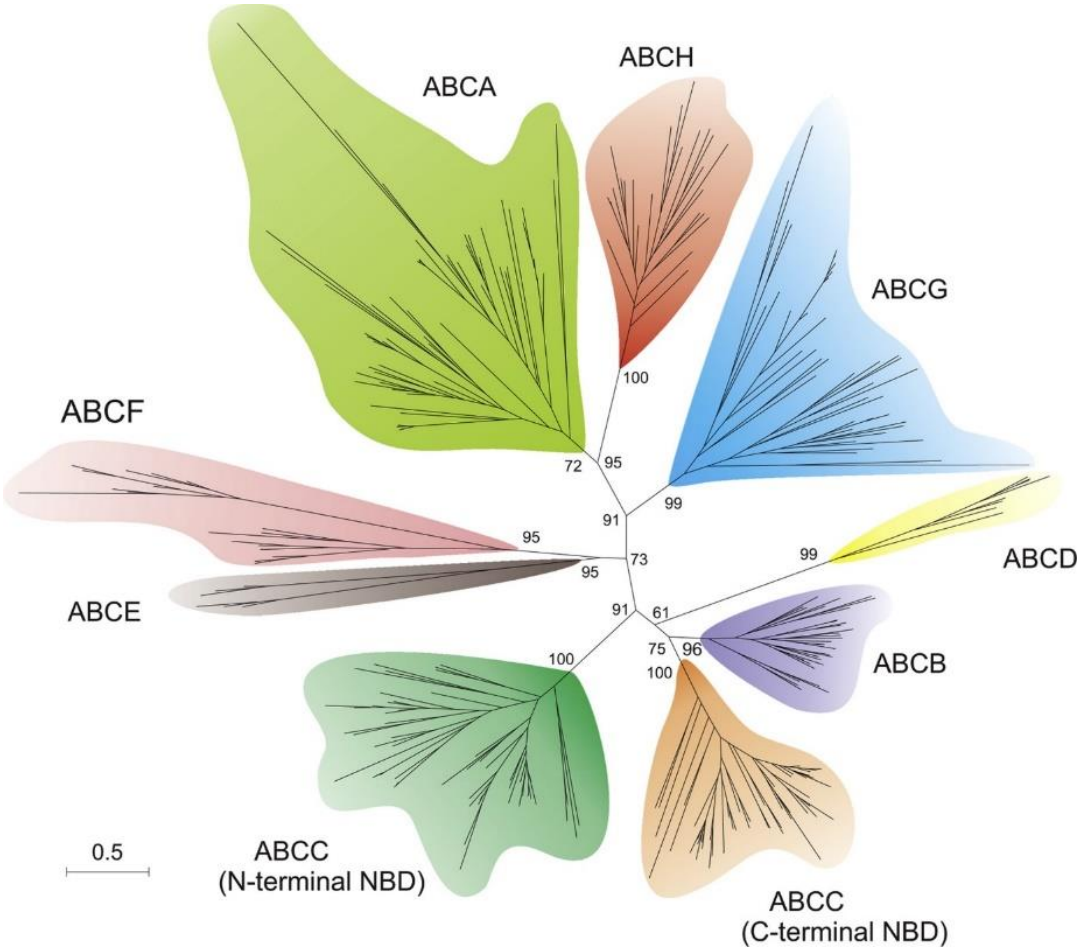


Figure 27. A schematic tree depicting the phylogenetic relationships between ABC subfamilies. NBDs of ABC proteins belonging to different subfamilies. C-terminal NBDs of the ABCC subfamily clustered together with NBDs of the ABCB subfamily. Adapted from Dermauw et al. *(2014) [268].*

ABC transporters have a ubiquitous distribution in tissues and cells. Their expression is variable during developmental stages and organism life cycle, as reported for sea urchins and mosquitoes. For example, mosquito egg and pupal stages show mainly ABCG and ABCC gene expression and nine ABC genes are expressed at a unique developmental stage [288, 289]. Interestingly, modifications in ABC gene expression occur even within few hours, as recently reported in larvae of the malaria vector *Aenopheles stephensi* [290].

The first discovered ABC transporter was the ABCB1 and was described by Juliano and Ling in 1976. They called the surface protein "P-glycoprotein", since it appeared to be associated with mutant Chinese Hamster Ovary cells displaying altered drug permeability [291]. The effect of this publication was an increasing interest in ABC transporters throughout the last three decades. Huge efforts were spent in understanding their biological functions and mechanisms, mainly due to their principal role in different diseases, including cancer, cholestasis, and cystic fibrosis, and the resistance to xenotoxins [272, 292, 293]. For this reason, most of the functional

information about these transporters pertains to the human transporters ABCB, ABCC, and ABCG "multidrug efflux" subfamilies. For example, increased expression of ABCB1, also known as P-gp (P-glycoprotein, for their role in permeability efflux modulation), ABCC1 (or MRP1, Multidrug Resistance Protein 1), and ABCG2 (or BCRP, Breast cancer resistance protein) is responsible for acquired drug resistance [294]. On the other hand, reduced expression of the transporters ABCB11 (or BSEP, Bile Salt Export Pump) and ABCC7 (or CFTR, Cystic Fibrosis Transmembrane Conductance Regulator) leads to cholestasis and cystic fibrosis, respectively [285, 294].

ABC transporters in arthropods

Compared to bacterial, nematode and human ABC transporters, the knowledge about arthropod ABC transporters is still limited. Although in the last few years more than 400 arthropod ABC proteins have been identified and more than 60 arthropod genomes have been sequenced, only the ABC transporters belonging to a few species have been widely studied. Among these, the red flour beetle *Tribolium castaneum*, the polyphagous spider mite *Tetranychus urticae*, the silkworm *Bombyx mori*, the mosquitoes *Anopheles gambiae*, *Aedes aegypti*, and *Culex pipiens quinquefasciatus*, and the water flea *Daphnia pulex* [288, 295-300]. The number of ABC transporters vary widely among orders, and often among families. For example, mosquitoes show a variable number of functional proteins, comprised between 55 (in *A. gambie*) and 69-70 (in *A. aegypti* and *C. p. quinquefasciatus*, respectively) [288]. However, only a few arthropod transporters have been described in detail. The involvement of ABC transporters in xenobiotic resistance in arthropods is historically not well documented, but an increasing number of studies are now emphasizing their importance [268].

ABCA

A clear orthologous relationship among the few arthropod ABCA subfamilies identified can be deduced from phylogenetic analysis, but their function is not completely clear. In humans, ABCA transporters are divided into 2 groups: the first includes ABCA1 to ABCA4, ABCA7, ABCA12 and ABCA13, while the second one includes ABCA5, ABCA6, ABCA8, ABCA9 and ABCA10. Most of them are incompletely characterised. The human ortholog ABCA1 is involved in disorders of cholesterol transport and HDL; ABCA3 has important roles in surfactant production; ABCA4 transports vitamin A derivatives in the outer segments of rod photoreceptor cells and therefore performs a crucial step in the vision cycle biosynthesis;

ABCA12 is responsible for keratinocyte lipid transport [285, 301, 302]. *D. pulex* presents four ABCA transporters, while mosquitoes have from 9 (*C. p. quinquefasciatus*) to 12 (*A. aegypti*) different coding genes [288], but little is known about their function.

Based on these few studied transporters, all the ABCA transporters are likely involved in lipid movements [301]. Moreover, a group of insect-specific ABCAs could be involved in insect development, as reported in *T. castaneum*, where silencing of two genes resulted in 30% mortality during the pupa-adult molt, as well as a severe wing defects [295].

ABCB

ABCB transporters include both full and half transporters and are the result of the evolution of lineage-specific duplications, as confirmed by their phylogenetical analysis [268]. The best known ABCB transporters belong to humans and *D. melanogaster*, although orthologues have been found in all arthopods, even in the previous three mosquito species, which share the same number of transporters, named ABCB1, ABCB6, ABCB7, ABCB8 and ABCB10 [288].

Full ABCB1 (P-gps) of insects are clustered in two main domains. Four full transporters belonging to this subfamily have been found in the *D. melanogaster* genome. The most important are mdr49, mdr50 and mdr65. The last protein is the principle constituent of the fruit fly blood-brain barrier, being involved in chemical and xenobiotic transport [303], while the mdr49 protein is prevalently involved in physiological processes, such as migration of stem cells and protection against neurodegeneration processes [304]. Moreover, they are modulated after chemical exposure, with mdr49 and mdr50 up regulated in response to polycyclic aromatic hydrocarbons and, in Malpighian tubules and gut, after methotrexate treatment [305-307]. Correspondences among human and arthropod ABCB genes are more evident in half transporters. Although no orthologues of human ABCB2, ABCB3 and ABCB9 have been found in arthropods, the presence of other three transporters has been confirmed. Human mitochondrial transporters ABCB6, ABCB8 and ABCB10 have at least one ortholog in insects, with *D. pulex* showing two ABCB10 co-orthologues. These three transporters have important roles in the biogenesis of cytosolic iron and sulphur clusters, heme biosynthesis, iron homeostasis and protection against oxidative stress [308]. It was suggested recently that human ABCB6 is located in cellular compartments other than the mitochondria, but the precise identity, substrate specificity and functionality has not yet been well determined [309]. Due to their deep ortholog relation, human and insect ABCB half transporter might share expression in mitochondria, as well as their functions.

Interestingly, *D. melanogaster* full transporters and other arthropod ABCB genes have frequently been related to insecticide transport and/or resistance, as in *A. aegypti* where *ABCB7* is up-regulated in a pyrethroid-resistant strain [269, 270]. In the same model, the expression level of the *ABCB1* transcript undergoes an eightfold increase 48 hours after temephos treatment. Selective inhibition of the transporter through a sublethal dose of verapamil, or through double-stranded RNA (dsRNA)-mediated gene silencing increases *A. aegypti* larvae mortality, demonstrating the involvement of P-gp expression in insecticide efflux [310].
Mastrantonio *et al.* in 2017 reported several ABCB gene modulations in a study on the malaria vector, *A. stephensi*. *ABCB2* and *ABCB6* are statistically up regulated following permethrin treatment. Interestingly, females reach the peak of expression within one hour post treatment, while males reach the same expression level within 24 hours. *ABCB3* and *ABCB4* were not modulated by the pyrethroid [311].

ABCC

Transporters belonging to this family are full transporters (at least in humans) involved in several different cellular processes. Some of them have an extra N-terminal transmembrane spanning domain (TM_0). Therefore, they are divided into "long" and "short" transporters [268]. Their main function is probably related to the export of a wide range of substrates with broad specificity, such as drugs, cyclic nucleotides, and endogenous/exogenous compounds, even partially metabolised and conjugated to glutathione [285, 312-314]. For these reasons, ABCC transporters are known as MRPs (Multidrug-Resistance Proteins). The first identified MRP was the human ABCC1 in multidrug resistant lung cancer cell line [315].
However, they are also involved in ion transport and/or in cellular receptor activity (e.g. in humans, ABCC7 is responsible for the efflux of Cl^- ions in several organs and mutations in the amino acidic sequence is related to the cystic fibrosis, while ABCC8 and ABCC9 are sulfonylurea receptors). Differently from ABCB transporters, ABCCs are able to recognise a broad range of glucoronate-, sulphate- and glutathione (GSH)-conjugated organic anions. Interestingly, their expression is often synergised with several phase II conjugating enzymes, such as the GSH-S-transferases (GSTs) and the UDP-glycosyltransferases (UGTs), increasing the possibility of resistance against drugs and carcinogens [316-318]. Evolutionary analysis revealed two orthologous relationships between human and arthropod ABCCs. The first is the human ABCC10/MRP7, whose function is not completely understood. However, the presence in both humans and all studied arthropods suggests a more conserved function [268]. The second orthologous class of conserved receptor is the sulfonylurea receptors (human ABCC8

and 9), involved in multiple physiological processes. Among these, glucose homeostasis, ischemic protection and innate immunity are the most important [319, 320]. This family seems to be missing in coleopterans, even if other ABC transporters may compensate for the lack. Moreover, other ABCC proteins in *D. pulex* and *Apis melifera* have been clustered with another group of the "short" human MRPs [268].

In mosquitoes, this is the second largest subfamily with 21, 18 and 15 genes in *A. aegypti, C. p. quinquefascaitus*, and *A. gambie*, respectively. Most of them cluster with *ABCC4*, but even with *ABCC1* and *ABCC3*. Together with *ABCC10*, they are involved in multidrug resistance, as demonstrated for *A. aegypti ABCC4* up regulated in pyrethroid-resistant strains [269]. On the other hand, *D. pulex* genome shows only seven different ABCC transporters.

Moreover, it has been shown that the *Drosophila melanogaster CG6214* gene, also known as *dMRP*, shares the same biochemical properties of the human ortholog ABCC1, able to recognise estradiol, glucuronide, leukotriene and other molecules. Interestingly, alternative splicing of the *dMRP* results in a functional diversity of the transporter [321]. In fact, exposure to chemotherapeutical agents, such as methotrexate, piperonylbutoxide, or ivermectin, resulted in ABCC transporter expression modulation, as reported in *D. melanogaster* dMRP and in *Pediculus humanus humanus* ABCC1 (*PhABCC4*) [305, 322]. Interestingly, permethrin treatment in *A. stephensi* induces an *ABCC11* down regulation within the first hour, no more statistically significant after 24 hours [311].

ABCD

The ABCD subfamily consists of half transporters, whose function seems to be highly conserved among species, as proved by the clear orthologous relationship between human and arthropod transporters [268]. Human genome encodes for four ABCD genes and functional dimers are located in peroxisome membranes and involved in the transport of long and branched chain acyl-CoA molecules [323]. Differently from humans, all insects have two ABCD genes, except for *D. pulex*, which genome encodes for three different transporters of this family, with homology to human ones ranging from 46% to 55%. However, their role in insects has not been completely understood.

ABCE and ABCF

Transporters belonging to these families share high conserved motifs across diverse taxa. They seem to have analogous functions among humans and arthropods and are involved in biological processes rather than in transport, because they lack of the transmembrane domains. In

particular, most eukaryotes possess only one ABCE protein. In particular, ABCE1 protein has been found in all eukaryotes examined to date and play an important role in ribosome biogenesis and translation regulation [324, 325]. It might have a crucial role in development, as reported in *T. castaneum*.[326] In addition to the conserved ABCE1 orthologous, an ABCE1-like paralogous group exists in mosquitoes, with unclear biological significance [288].
ABCF, on the other hand, is probably only involved in translation regulation [326]. Mosquitoes have three ABCF proteins, orthologous to ABCF1, ABCF2, and ABCF3, while *D. pulex* presents four [288, 299].

ABCG

The ABCG family is also highly conserved among kingdoms and to date has been found in all the examined fungi, plants and metazoan species. The only reported difference is linked to the physiology of transporters, because in metazoan have been reported only half transporters, while in plants and fungi have been identified even full transporters, also named PDR (Pleiotropic Drug Resistance) proteins [327, 328]. Differently from other ABC proteins, which show a common structure summarised in Figure 26 (A), ABCG half transporters have a reverse domain organization, with the nucleotide binding fold localized in the N-terminal side of the transmembrane domain.
The human genome encodes for five ABCG transporters. Four of them are involved in the transport of endogenous and dietary lipids, limiting the absorption of plant sterols and cholesterol introduced with diet and promoting secretion of plant sterols and cholesterol from liver cells into the bile [329-331]. The last human ABCG transporter is the ABCG2, also known as BCRP (Breast Cancer Resistance Protein), which does not have a strict substrate specificity and is known as a multidrug efflux pump [330, 331]. The comparative analysis of ABCG gene family, conducted by Dermauw and Van Leeuwen in 2014, revealed the presence of ortholog proteins in each arthropod species under investigation, allowing the hypothesis that they could have similar role as their human orthologues [268]. The most studied arthropod ABCG genes belong to *D. melanogaster*, which are involved in the uptake of pigment precursors in cells of Malpighian tubules and developing compound eyes. Mutations in ABCG genes are associated with different eye-colour phenotype, i.e. white, scarlet, and brown, instead of the red-brown wildtype [332]. Even *T. castaneum* and *B. mori* orthologs have been related to eye pigmentation [295, 333]. However, the complete role of *D. melanogaster* ABCG genes is not yet completely clarified, since they seem to be involved in many other functions [333-336]. As well, the transporters of the other arthropods might also have alternative functions. For example, the gene

CG3327 of *D. melanogaster*, also named *E23* (*Early gene at 23*), and orthologues in other species are activated by the steroid hormone ecdysone, which play an essential role in insects, coordinating developmental transition such as larval moulting and metamorphosis [295, 297, 337]. In mosquitoes and *D. pulex*, this is the largest subfamily of ABC transporters, with an important expansion in *C. p. quinquefasciatus*. However, their functions are not yet completely known [288, 299].

In arthropods, many ABCG transporters are induced by ecdysone, suggesting that they might be involved in development. However, ABCG transporters are important in growth, as they are involved in lipid transport to the cuticle. This step is essential for the formation of a waterproof barrier in the epicuticle, as reported by Broehan *et al.* (2013) in larvae of *T. castaneum* [295].

The lack of close-related orthologues to human ABCG2 in arthropods is interesting, although some of them respond to xenobiotic exposure, as reported in *A. stephensi*, where *ABCG4* expression is up regulated within the first hour of treatment with permethrin and is still significant after 24 hours [296, 311]. For this reason, arthropod ABCG transporter might play an important role in xenobiotic resistance [270, 338].

ABCH

Similarly to ABCGs, this most recently described class is made up of half-transporters with a reverse domain structure. As previously mentioned, the ABCH subfamily has been identified only in arthropods and zebrafish genome, while it is lacking in mammals, plants and fungi [285, 327, 328]. Arthropods generally express three different ABCH transporters, but the number increases to four in *A. aegypti*, nine in the pea aphid *Acyrthosiphon pisum*, fifteen in *D. pulex*, and twenty-two in *T. urticae*. Although their function is not completely understood, it seems they are involved in development and in controlling of diapause period in *T. urticae* females, as well as in the transport of cuticular lipids, which are deposited in the outer epicuticular layer to prevent water loss [288, 299, 339]. A particular study in two substrains of *Plutella xylostella*, a basal lepidopteran species, highlights the potential role of ABCH transporters in detoxification and xenobiotic resistance, in particular to chlorpyrifos and fipronil. [268, 340].

ABC transporters in ticks

As previously described, ABC transporters play an important role in many metabolic pathways. A study conducted by Mulenga *et al.* in 2007 reported the up-regulation of a putative ABC

transporter implicated in peptide transport in *Amblyomma americanum* ticks, during the attachment phase to hosts and before skin penetration [341].

To date, only a few studies have focused attention on the involvement of these transporters in detoxification processes against pesticides.

Pohl *et al.* described the involvement of ABC transporter proteins from both ABCB and ABCC subfamilies following ivermectin exposure in two strains of *Rhipicephalus* (*Boophilus*) *microplus*, the first sensible, while the second resistant to the macrocyclic lactone. The expression of the RmABCB10 transporter significantly increased in ivermectin-resistant females, compared with susceptible ones. Moreover, the expression rose to 2-fold in response to ivermectin feeding, compared to untreated ticks. Exposure to sub-lethal doses of ABC inhibitors, Cyclosporin A (CsA) and MK571, partially restored the sensitivity to the molecule. Interestingly, the same inhibitors had different effects on F1 and F3 generations, indicating the development of different resistant genotypes due to drug selection pressure. They can be related to insensitivity of the glutamate-gated chloride channels, or to an increase in metabolic detoxification mediated by esterases, cytochrome P450 monooxygenase and glutathione-*S*-transferase [29, 30].

Ticks digest host haemoglobin in the digestive vesicles (similar to lysosomes) and then accumulate heme aggregates into specialised organelles, called hemosomes. Lara *et al.* described the involvement of the RmABCB10 protein in amitraz-resistance mechanisms, using *Rhipicephalus* (*Boophilus*) *microplus* midgut cells. They demonstrated the presence of RmABCB10 transporter on the surface membrane of hemosomes and the accumulation of amitraz into the same organelles. Moreover, analysis of *RmABCB10* transcription levels in digest cells revealed significant higher expression of the transporter in the amitraz-resistant strain compared to sensitive one, confirming its involvement in detoxification processes [342].

The study of Cafarchia *et al.* in 2015, confirmed the potential role of ABC transporters in the dog tick *Rhipicephalus sanguineus* sensu lato. Larvae were treated with fipronil and ivermectin, and mortality rates were compared to those deriving from the combination of the drugs with a sub-lethal dose of CsA. Results showed an increase in mortality rates of about 14-fold for fipronil and 22-fold for ivermectin [31].

There are very few studies describing ABC transporters in tick cell lines. The first, published in 2014 by Pohl *et al.*, showed that ABC transporters play an important role in the development of resistance to ivermectin in the *Rhipicephalus microplus* cell line, BME26. In particular, the *RmABCC1*, *RmABCB7*, and *RmABCB10* genes were up-regulated in treated cells. Furthermore, in the same study, authors were able to establish a resistant sub-culture (BME26-IVM), through

treatments with increasing doses of ivermectin, starting at 0.5 μg/ml and ultimately reaching 6 μg/ml. Incubation with CsA partially restore the sensitivity to ivermectin, confirming the involvement of ABC transporter in drug resistance [34].

Koh-Tan *et al.* in 2016 examined several tick cell lines deriving from different genera (*Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Ixodes*) in order to study two genes implicated in drug resistance mechanisms. The first encoded for the β-adrenergic octopamine receptor, and previous studies described its involving in resistance to amitraz [22]. The second for the ABCB10 transporter. Interestingly, the *Rhipicephalus microplus* cell line, BME/CTVM6, deriving from a resistant strain, expressed a novel β-adrenergic octopamine receptor-like gene, and a significantly high level of the *ABCB10* gene [33].

However, a common hypothesis is that different ABC transporter proteins may have different degrees of relevance in drug detoxification during larval or adult stages.

4. Experimental study of *ABCB* gene expression in an *Ixodes ricinus* continuous cell line exposed to acaricides

Materials and Methods

IRE/CTVM19 line maintenance

The IRE/CTVM19 cell line is derived from the embryonic stage of *I. ricinus* [32]. Cells were maintained according to guidelines developed by L. Bell-Sakyi. The cell line was grown in flat-sided tubes (Nunc™, Thermo Scientific, Milan, Italy) and incubated at 28°C in 2 ml of Leibovitz's L-15 medium (Life Technologies, Milan, Italy) supplemented with 20% fetal bovine serum, 10% tryptose phosphate broth, 2mM L glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Life Technologies, Milan, Italy).

Medium was replaced every seven days and cells were sub-cultured at least every 15 days. For medium change, tubes were left for 5 to 10 minutes vertically and then 1.5 ml of exhausted medium was replaced with the same amount of fresh medium. For sub-culturing, on the day of medium change, the removed exhausted medium was placed overnight into a new tube for the conditioning of the plastic material. The day after, the exhausted medium was discarded from the new tube, 2 ml of fresh medium was added to the parental cell population, which was re-suspended and, finally, 2 ml of the cell suspension was transferred in the new tube.

Growth curve analysis

For growth rate analysis, interpreted as doubling time of cells in culture, two series of four tubes each were followed for five weeks. One series differed from the other in the number of initial seeded cells ($1x10^6$ cells/ml or $2x10^6$ cells/ml). Every seven days, in occurring of medium change, a small aliquot of cell suspension was harvested from each tube, labelled with Trypan Blue 0.4% w/v (Sigma-Aldrich, Milan, Italy) and counted using a haemocytometer.

Treatments of IRE/CTVM19 cells

Experiment 1.

Treatment of IRE/CTVM19 cells with low, increasing concentrations of ivermectin

IRE/CTVM19 cells deriving from cultures at the same passage level were centrifuged, and re-suspended in fresh L-15 medium at a concentration of $1x10^6$ cells/ml. New tubes were filled each with two ml of the cell suspension, and immediately treated with different concentrations of ivermectin (IVM, Sigma-Aldrich, Milan, Italy), previously re-suspended in dimethyl sulfoxide (DMSO). Three concentrations of drug were selected, basing on the previous study of Pohl *et al.* [34]. However, higher doses of ivermectin were selected, based on the low level of *I. ricinus* methabolism: 11 μM, 22 μM or 33 μM. For each condition, the final percentage of DMSO was maintained at 0.1%. Two controls were taken into account and represented by untreated cells and cells treated with 0.1% DMSO. Tubes were incubated for 10 days and medium was changed weekly, maintaining the same concentrations of ivermectin. To avoid cell loss, tubes were centrifuged at 300 x g for 5 minutes and then medium was changed as previously described.

Experiments were carried out with four replicates per treatment protocol.

Experiment 2.

Treatment of IRE/CTVM19 cells with high, increasing concentrations of amitraz, fipronil, permethrin, and ivermectin

As previously described, cells deriving from cultures at the same passage level were centrifuged, re-suspended in fresh complete L-15 medium to a final concentration of $1x10^6$ cells/ml. Two ml of the cell suspension were seeded into new tubes, and treated with a range of concentrations of analytical standard amitraz (AMZ, Sigma-Aldrich, Milan, Italy), fipronil (FIP, Sigma-Aldrich), or permethrin (PERM, Sigma-Aldrich). Drugs were previously dissolved in DMSO and then diluted in complete culture medium to final concentrations of 25 μM, 50 μM, 100 μM and 150 μM, according to previous studies on different cell lines (i.e. human neuroblastoma SH-SY5Y, rat adrenal pheochromocytoma PC12, and porcine kidney IB-RS-2). The DMSO concentration was kept unvaried at 0.5%. Control samples were left untreated, or treated with 0.5% DMSO only. Cultures were maintained for 10 days and medium was changed

once on day seven. Tubes were centrifuged at 300 x g for 5 minutes to harvest all cells in suspension and then medium was changed.

Experiments were carried out with four replicates per treatment protocol.

In order to create comparable data between molecules, treatment with ivermectin was repeated with higher doses of ectoparasiticide. IRE/CTVM19 cells of the same passage level were harvested, centrifuged, re-suspended in fresh medium, and seeded at a concentration of $1x10^6$ cells/ml in 2 ml culture medium per tube. They were treated immediately with the same doses of ivermectin as the previous drugs (25 µM, 50 µM, 100 µM and 150 µM). The final concentration of DMSO was 0.5%. Untreated cells and cells treated with 0.5% DMSO only served as controls. Tubes were incubated for 10 days and medium was changed on the seventh day, as previously described. New media contained the same concentrations of ivermectin as reported above.

Cell viability, morphology and cell density

Experiment 1

Flow-cytometry and Trypan Blue assay

A trial comparison of cell viability was performed on day 5 of the first treatment of the experiment 1, using the LIVE/DEAD™ Fixable Near-IR stain kit (Life Technologies, Milan, Italy) and Trypan Blue 0.4% w/v (Sigma-Aldrich, Milan, Italy).

For LIVE/DEAD™ Fixable Near-IR analysis, cells were stained according the manufacturer's instructions and analysed by flow-cytometry. Flow-cytometry was performed using a BD FACSVerse (BD Biosciences, Stockholm, Sweden) equipped with 488 nm blue and 633 nm red lasers, and results were analysed using the FACSDiva (BD Biosciences) software. Cells frozen at -80°C and thawed three times were used as negative controls.

For the Trypan Blue assay, a small aliquot of cell suspension was harvested from each tube, labelled with Trypan Blue 0.4% w/v (1:1 ratio), and counted using a haemocytometer, as previously reported by Strober W. in 2015 [343].

Subsequent measurements were performed on day 10 of each treatment.

Experiment 2

Trypan Blue and MTT assays

As the flow-cytometry procedure required a large number of cells, subsequent analysis were performed only with Trypan Blue assay, as previously described. Labelled, small aliquots of the same cell suspension were used in order to evaluate the number of viable cells and their density.

At the same time, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich, Milan, Italy) assays were also carried out at the end of each treatment. One hundred µl of the resuspended cells were transferred in a 96-well plate. MTT powder was dissolved in L-15 complete medium at a concentration of 5 mg/ml, and 10 µl of the final solution were added to each well. Plates were incubated at 28°C for 3 hours, then centrifuged at 300 x g for 5 minutes to harvest possible cells in suspension. Medium was carefully removed and replaced with 100 µl of a lysis solution containing 10% w/v SDS (Sodium Dodecyl Sulfate) and 10 mM HCl. Plates were incubated overnight and the day after the absorbance at 570 nm was measured with a Victor3 V plate reader (Perkin Elmer, Milan, Italy) normalizing the values against the absorbance at 650 nm. The mean of three independent experiments was calculated and each condition was compared to the untreated control.

Cell morphology

In experiment 2, after 10 days of incubation, cell morphology was examined in smears prepared with approximately 20 µl of cell suspension and dried under the hood flow. Later, slides were stained with MayGrunwald Giemsa and observed at different magnifications through an Eclipse Ci-L microscope (Nikon, Italy). Cell imagines were captured through a 5-megapixel Digital Sight DS-Fi1 camera (Nikon, Italy), which captures at a high resolution of 2560 x 1920 pixels.

RNA extraction and gene expression profile after acaricide treatment

RNA extraction and cDNA synthesis

On day 10 following the beginning of every treatment, RNA was extracted from each replicate of samples using an RNeasy Mini Kit (Qiagen, Milan, Italy). Firstly, tubes were centrifuged 300 x g for 5 minutes to harvest all cells, washed twice with PBS (Phosphate Buffered Solution) to remove all medium traces and then processed following the manufacturer's instructions.

Extracted RNA was measured by spectrophotometer analysis for quality and quantity assessment, and then 300 ng were converted into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad, Milan, Italy), according to manufacturer's instructions. The resultant cDNAs were used as template for downstream molecular analysis.

Primer design and validation

Before of this study, no information were available about *I. ricinus* sequences for any of the transporters under investigation (*ABCB1*, *ABCB6*, *ABCB8* and *ABCB10* genes); thus primers were designed, basing on conserved regions shared between selected ABC transporters of *I. scapularis* available in genbank [344] and the *Rhipicephalus sanguineus* transcriptomes, kindly given by S. Epis (University of Milan, personal communication). As endogenous control, *β-actin* gene was chosen and primers were designed based on the partial sequence available in genbank (HQ682101) (Table 7).

Table 7. List of designed primers used for the analysis of ABC gene and β-Actin *expressions.*

	Primer Sequences	Predicted length (bp)
ABCB1	F: 5' - TCTTTGCCGTCTTCTACAG - 3' R: 5' - CAGGTTCTCTCCAGCGAT - 3'	167
ABCB6	F: 5' - AGACTATGTCCTCTTCCTCA - 3' R: 5' - CATCTATCACCTCTGCCTT - 3'	146
ABCB8	F: 5' - ATCAGGAACGCCGACATC - 3' R: 5' - AGTTTCCAGTAGACACCCTT - 3'	101
ABCB10	F: 5' - TGTCCTAACCATTGCTCACA - 3' R: 5' - TGATGTTCCACTAATGTCCG - 3'	147
β-Actin	F: 5' - CACGGCATCGTGACCAACTG - 3' R: 5' - CGAACATGATCTGAGTCATCTTCTC - 3'	157

Primer specificities were tested in a traditional PCR (Polymerase Chain Reaction) using cDNA derived from untreated IRE/CTVM19 cultures. The optimised amplification protocol was run on a C1000 thermal cycler (Bio-Rad, Milan, Italy), and characterised by a denaturation step at 95°C for 5 min, followed by 45 repeated cycles (95°C – 10 sec; 56°C – 15 sec; 72°C – 15 sec). A final elongation step at 72°C was introduced for 5min. The final concentration of each primer in all the reactions was 0.4 µM. Reactions were run on a 2% agarose gel stained with SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Milan, Italy) and examined under UV light (UView mini Transilluminator, Bio-Rad). The obtained amplification fragments were purified

through a QIAquick PCR Purification Kit (Qiagen, Milan, Italy), according to manufacturer's instructions, and later sequenced in order to confirm their specificity.

Real-Time PCR

Levels of expression of *ABCB1*, *ABCB6*, *ABCB8*, *ABCB10* genes, as well as the endogenous control β-actin, were detected through quantitative RT-PCR. Different kits and instruments have been used in the treatment series.

Total mRNAs extracted from experiment 1 were analysed using the SYBR Green master mix kit (EuroClone, Milan, Italy) on a Stratagene Mx3005p machine (Agilent Technologies, Milan, Italy), according to the manufacturer's instructions. Amplification protocol and primer concentration were the same as previously described. Fluorescence signals were captured at the end of every cycle and the analysis of melting curves avoided the presence of nonspecific products. Results were analysed through MxPro QPCR Software (Agilent) and results expressed as Relative Normalised Expression (ΔΔCq).

Quantitative RT-PCRs in the experiment 2 were performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad), and according to the manufacturer's instructions. Changing in master mix and instrument needed of a further optimisation of the protocol. The optimal concentration of each primer in all reactions was 0.25 µM, while the amplification protocol so changed: a denaturation step at 98°C for 2 min, followed by 45 repeated cycles (98°C for 10 sec; 57°C for 15 sec; 72°C for 20 sec). Fluorescence signals were collected at the end of every cycle and the presence of nonspecific products was excluded through analysis of the melting curves. Results were analysed by CFX Manager software (Bio-Rad) and results expressed as Relative Normalised Expression (ΔΔCq).

Data analysis

The means of three independent sessions for each experiment with four replicates each were used to detect statistical significance in a one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test on Past3 (http://folk.uio.no/ohammer/past/, v3.14). A p-value <0.05 was considered statistically significant.

IRE/CTVM19 transcriptome

Transcriptome analysis was carried out on cells treated with fipronil at 100 µM, and on untreated and DMSO-treated controls, for a preliminary study on the mechanisms involved in fipronil detoxification. Cells deriving from the same passage number were centrifuged, re-suspended in fresh medium to a final concentration of 10^6 cells/ml, seeded in new tubes, and treated as previously described. Four different tubes were used for each control condition, while ten tubes were used for fipronil treatment. Following 10 days of treatment, cells were harvested and total RNA was extracted using the PureZOL™ RNA isolation reagent (Bio-Rad), according to the manufacturer's instructions. RNA quantity was determined through a NanoDrop™ 2000 spectrophotometer (Thermo Fisher) and quality checked through a 2100 Bioanalyzer (Agilent) instrument. Only samples with high quality mRNA were sent for sequencing (Parco Tecnologico Padano, Lodi, Italy).

The RNA-sequencing service included a TruseqRNA library preparation, and sequencing of fragments on an Illumina Hiseq2500 platform (San Diego, USA) with a 100PE module.

Data were immediately downloaded, unzipped and processed on an external server with the latest Ubuntu OS and equipped with appropriate hardware (in terms of hard disk capacity and number of available processors). Data analyses were carried on at the University of Cambridge, under the supervision of the Dr. Cinzia Cantacessi.

The first step of sequence data analyses was a check of raw data quality using the *FastQC* program. The presence of rRNA has been removed through the *SortMeRNA* program, then the paired-end sequences were merged and the adapters were trimmed using the *Trimmomatic* program, followed by a second *FastQC* analysis.

All clean data were assembled using the *Trinity* program, and then normalised and filtered in order to remove unspecific clusters or less abundant transcripts. The applied filter was the 2.5 FPKM (fragments per kilobase transcript length per million fragments mapped).

The filtered transcripts were used as input for a Transcriptome Functional Annotation and Analysis (*Trinotate* program). *Trinotate* makes use of a number of different well referenced methods for functional annotation, including homology searches to known sequence data (BLAST+/SwissProt), protein domain identification (HMMER/PFAM), protein signal peptide and transmembrane domain prediction (signalP/tmHMM), and comparisons to data available in a range of annotation databases (eggNOG/GO/Kegg databases).

Results

Grow curve analysis

Growth curve analysis revealed a doubling-time of approximately ten days for IRE/CTVM19, independently of the initial amount of cells (Figure 28). This result might reflect the natural slow cycle of *I. ricinus* and led us to choose $1x10^6$ cells/ml as initial concentration for seeding, and 10 days as end-point for the treatments. Moreover, although tick cell lines do not exhibit contact inhibition, the maximum number of cells per tube was about 30 millions.

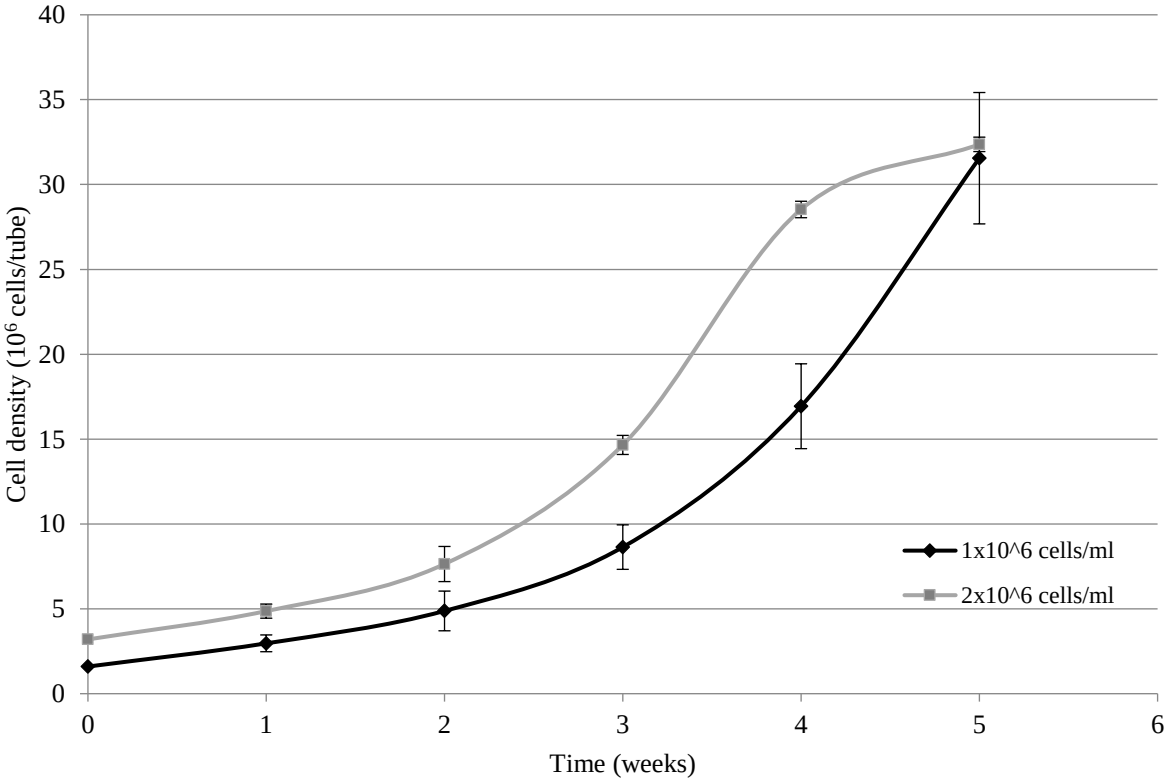


Figure 28. Growth curve for IRE/CTVM19 cell line. Two conditions have been evaluated: $1x10^6$ cells/ml (black curve) and $2x10^6$ cells/ml (grey curve).

Validation of primer couples

Traditional PCR revealed a single fragment for each primer couple previously described (Figure 29). For *ABCB10* gene, two different reverse primers were designed, but only the first, which generated a longer amplicon, was used for downstream analysis.

The sequenced fragments were deposited in EMBL Nucleotide Sequence Database (*ABCB1*: LT222035; *ABCB6*: LT222036; *ABCB8*: LT222037; *ABCB10*: LT222038), as reported in the first publication on this project [345].

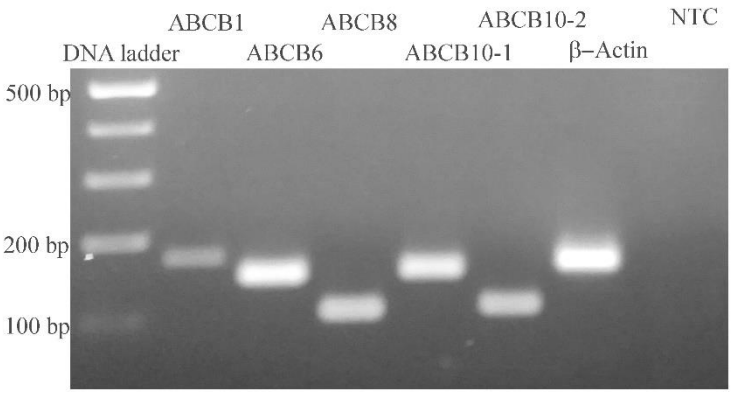


Figure 29. Traditional PCR for validation of primer couples. For ABCB10 gene, two different reverse primers were designed.

Experiment 1. Treatment of IRE/CTVM19 cells with low, increasing concentrations of ivermectin

Morphology and viability

IVM treatments resulted in a modification of cell morphology, with bigger and more vacuolated cells compared to controls. The adherence to the tube surface was also altered (Figure 30). However, viability analysis did not show any reduction in the number of live cells. Trypan Blue exclusion assay (data not shown) and flow-cytometry (Figure 31) gave similar results with 88-92% of live cells in the controls (untreated tubes, or tubes treated with DMSO), and 84-87% in cells treated with increasing doses of ivermectin. Statistical analysis did not highlight any significance difference among groups.

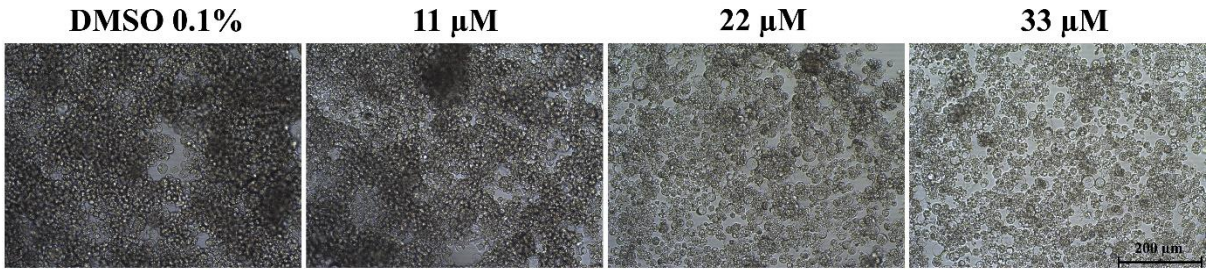


Figure 30. Alteration of cell adherence, morphology, and density after treatment with increasing doses of ivermectin. Pictures were captured using an IB2FL inverted microscope (Exacta Optech, Thame, UK) and OrmaEurotek camera (Orma Scientific, Milan, Italy) at 100X magnification; scale bar: 200 µm.

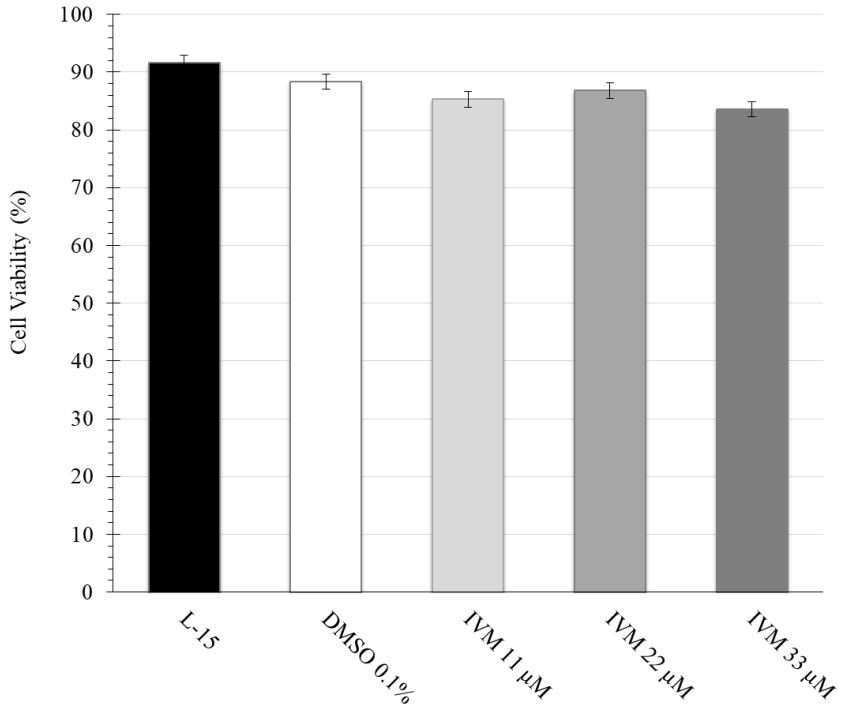


Figure 31. IRE/CTVM19 viability following treatment with increasing doses of ivermectin, measured through flow-cytometry. Statistical analysis did not evidence any difference among groups. Data were collected from three independent experiments, and expressed as absolute values of the mean ± SEM.

ABCB gene expression

Initial results in quantitative RT-PCR showed the expression of *ABC* genes in the IRE/CTVM19 cell line. However, statistical analysis showed few significant modulations following ivermectin treatment (Figure 32). Although *ABCB8* gene was up-regulated when cells were treated with ivermectin at 22 µM and *ABCB6* gene was up-regulated at higher concentrations, no one condition was statistically significant compared to the DMSO-treated control. *ABCB10* gene was expressed in every condition, but no differences among treatments were evident. Unfortunately, expression of *ABCB1* gene was not detectable at any time point in any condition (data not shown).

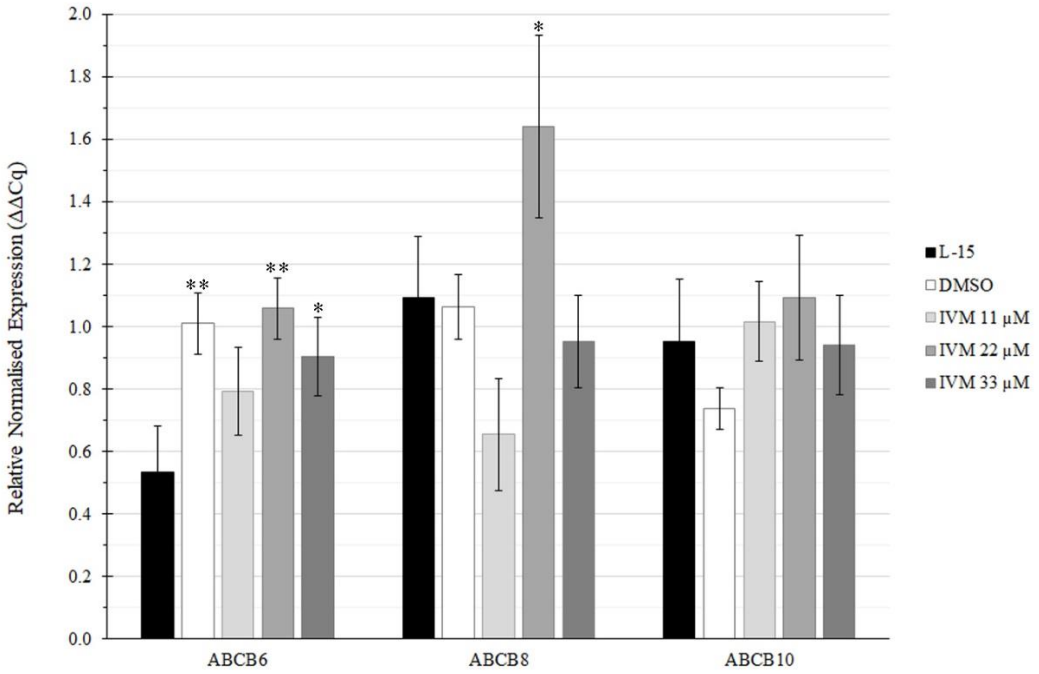


Figure 32. Expression of ABCB6, ABCB8 *and* ABCB10 *genes in IRE/CTVM19 cells treated with increasing doses of ivermectin. Results are reported as the mean of three independent experiments, with four replicates each, expressed as relative normalised expression (ΔΔCq) ± SEM. *p<0.05; **p<0.01*

Experiment 2. IRE/CTVM19 treatments with amitraz, fipronil, permethrin, and higher concentrations of ivermectin

Morphology

Macroscopic observation of treated IRE/CTVM19 tubes showed different patterns after 10 days of incubation. Tubes treated with amitraz and permethrin presented yellowish media at every concentration, sign of good levels of cell metabolism. On the contrary, treatments with fipronil and ivermectin induced alterations in the tube environment, as highlighted by the gradual change of the medium colour to pink (Figure 33).

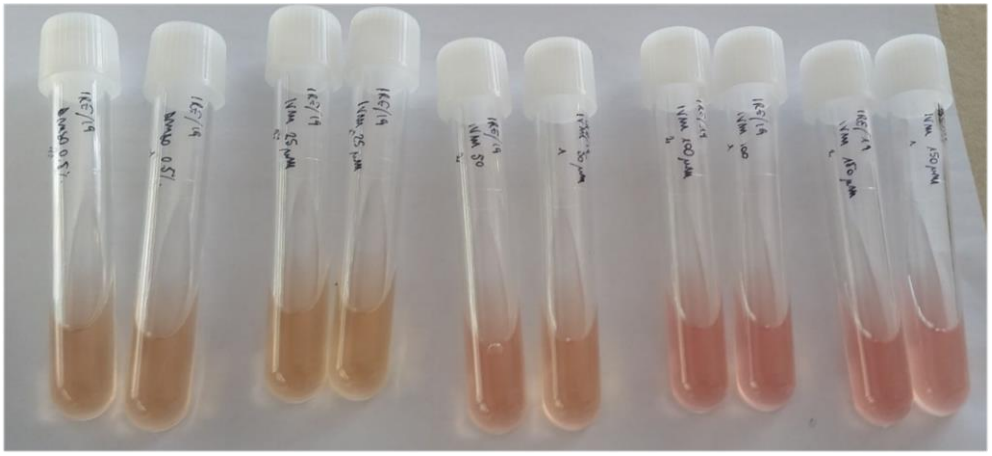


Figure 33. Example of macroscopic alterations induced by ivermectin treatment. IRE/CTVM19 cell metabolism caused an acidic environment (yellowish medium). Increasing drug concentrations altered cell metabolism, as confirmed by the medium colour shift to pink. In particular, the first two tubes per condition were used to take a demonstrative picture.

Effects of ectoparasiticides were evident even at microscopic levels, with modifications in cell adherence and morphology, as reported in Figure 35.

Moreover, all molecules induced alternations in cell morphology, especially at high concentrations (100-150 µM), as showed in Figure 34. Treated cells appeared shrunk, with pyknotic nuclei and karyorrhexis. Alterations were particularly evident with fipronil (Figure 34B) compared to untreated control (Figure 34A).

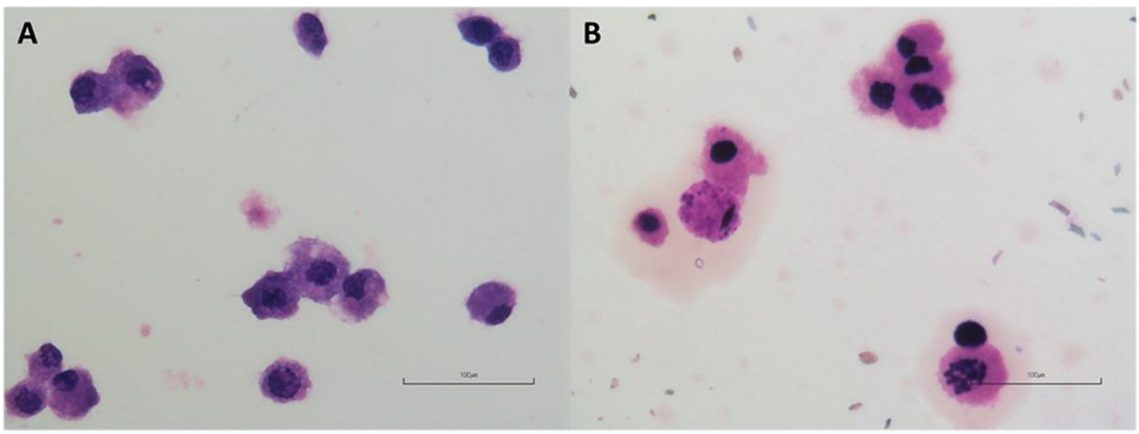


Figure 34. Cytological alterations following IRE/CTVM19 treatments. In particular, they were evident following fipronil treatment (B) compared to DMSO-treated control (A). Pictures were captured using an Eclipse Ci-L microscope (Nikon, Milan, Italy) and a Digital Sight DS-Fi1 camera (Nikon, Italy) at 1000X magnification; scale bar 100 µm.

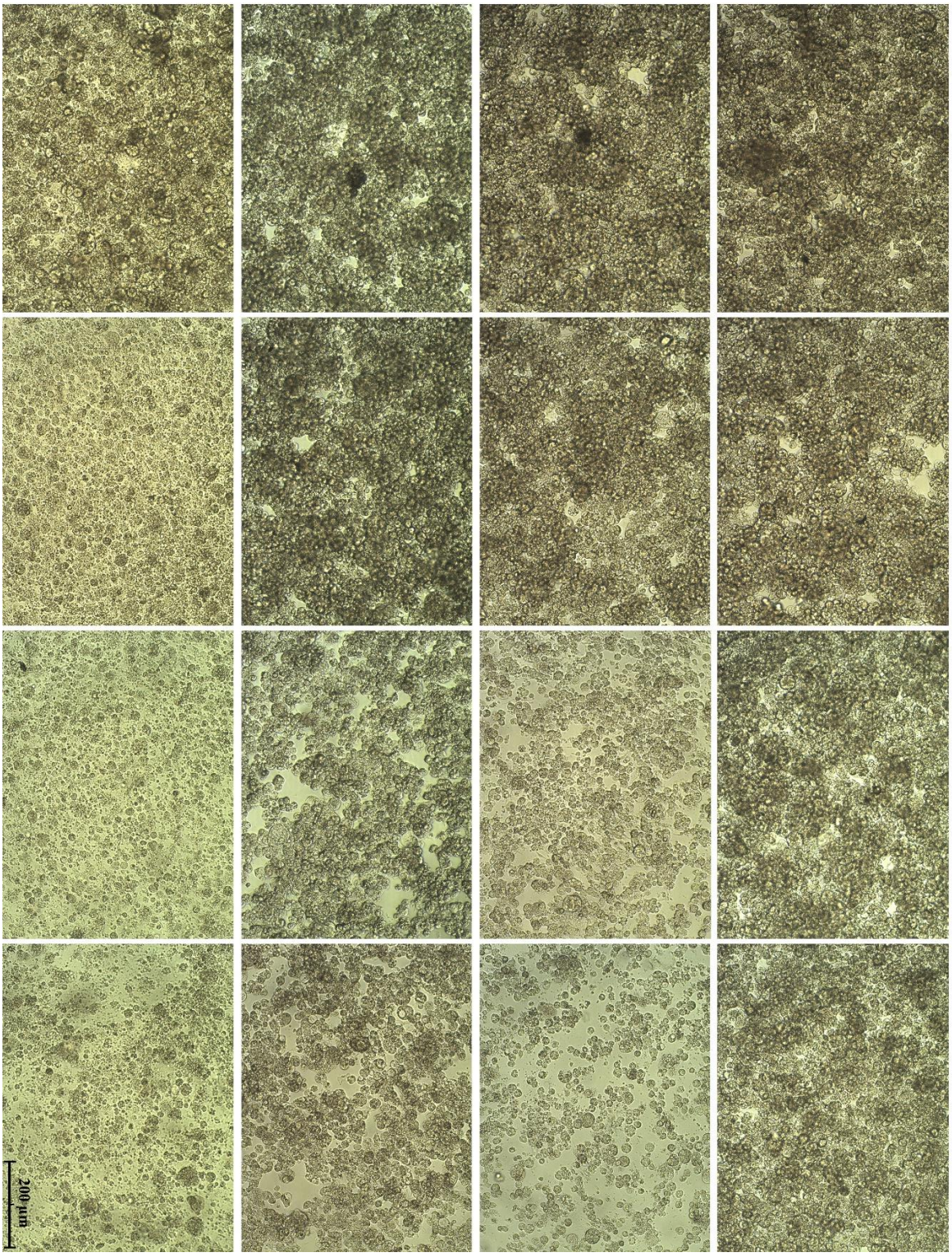


Figure 35. Alteration of cell adherence, morphology, and density after IRE/CTVM19 treatment with increasing doses of amitraz, fipronil, permethrin, and ivermectin. Pictures were captured using an IB2FL inverted microscope (Exacta Optech, Thame, UK) and OrmaEurotek camera (Orma Scientific, Milan, Italy) at 100X magnification; scale bar: 200 µm.

Viability

Trypan Blue exclusion tests and MTT assays generated discordant results concerning viability of cells (Table 8).

Analysis of Trypan Blue exclusion assays showed no significant difference in viability of cells treated with amitraz at any concentration, when compared to untreated cells, or cells treated with DMSO alone ($p>0.1$). However, cell density decreased when IRE/CTVM19 were treated with higher concentrations of amitraz, and was significant at 150 µM ($p<0.01$) (Figure 36 and Figure 37, light grey bars). Fipronil treatment induced a significant decrease in cell viability at 100 µM (up to 88.7%, $p<0.05$) and 150 µM (up to 63.2%, $p<0.01$) and in cell density (up to 42.9% at the highest concentration, $p<0.001$). Similarly to amitraz, cells treated with permethrin showed no significant decreases in cell viability at any concentration ($p>0.1$), but cell density was negatively affected up to 47.5% at 150 µM ($p<0.001$) (Figure 37, light grey bars). Ivermectin treatments led to a reduction in cell viability with increasing doses of ectoparasiticide: 50 µM reduced viable cells up to 88.1% ($p<0.05$), while 100 µM up to 77.9% ($p<0.005$), and 150 µM up to 58% ($p<0.001$). Even cell density was progressively reduced up to 53% at 150 µM ($p<0.001$) (Figure 37, light grey bars).

Viability results from MTT assays (Figure 36 and Figure 37, dark grey bars) diverged from Trypan Blue exclusion results in all the treatments, except for amitraz up to 100 µM. The highest amitraz concentration, 150 µM, induced a significant lowering in MTT salt reduction compared to untreated cells, or cells treated with DMSO. Fipronil, permethrin, and ivermectin induced significant decrease in formazan synthesis ($p<0.001$) at all drug concentrations. Interestingly, fipronil showed a dose-dependent reduction up to 6.6% at the highest concentration, while ivermectin drastically down-regulated metabolising cells up to 40% at 25 µM, with imperceptible signals at higher doses (Figure 37).

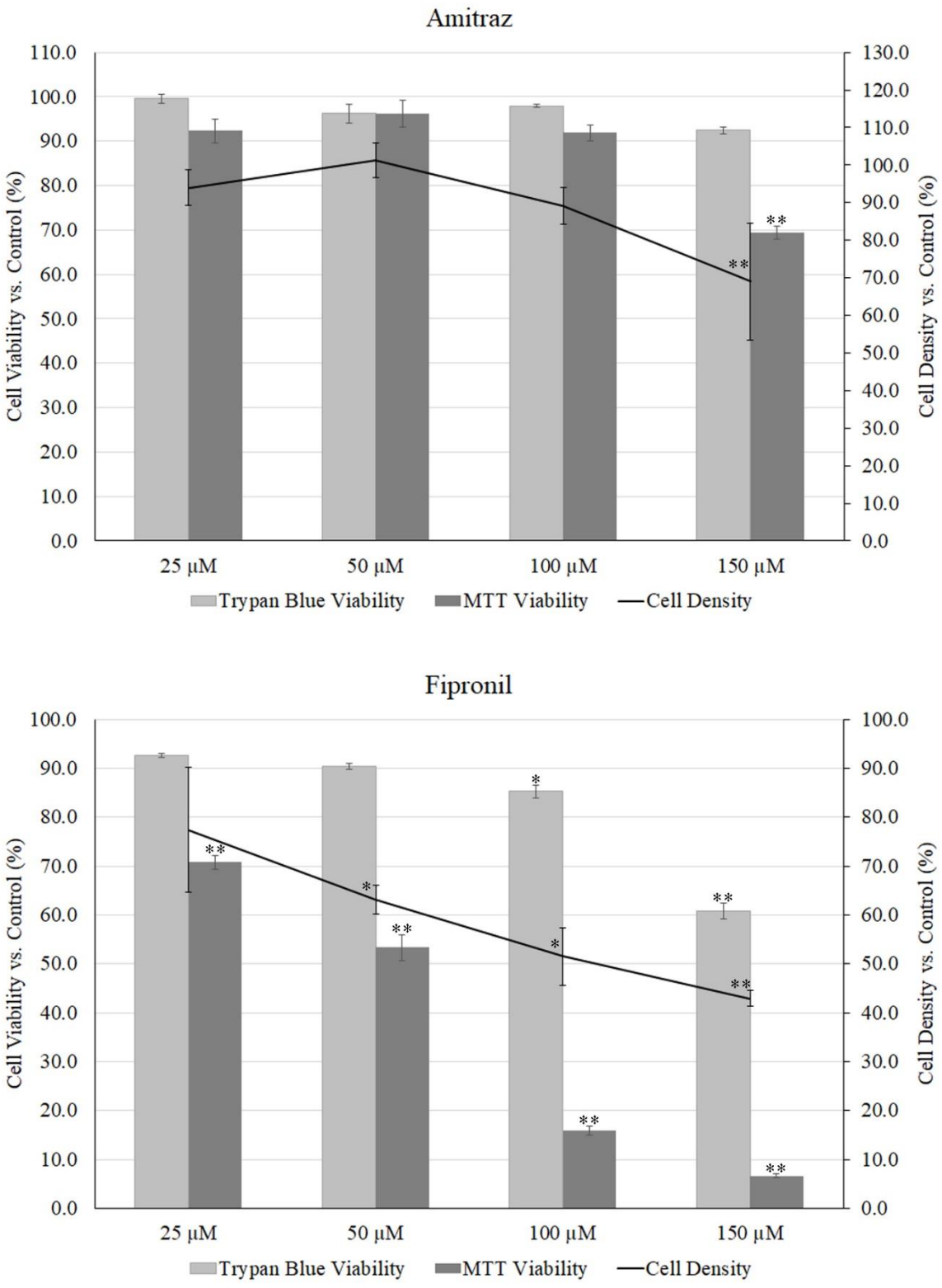


*Figure 36. IRE/CTVM19 cell viability with Trypan blue (light gray bars), MTT assays (dark gray bars), and cell density (black line) following treatment with increasing doses of amitraz (top) and fipronil.(bottom) Data were reported as the mean of three independent experiments, with four replicates each, expressed as percentage of the control (0.5% DMSO) ± SEM. *p<0.05; **p<0.01*

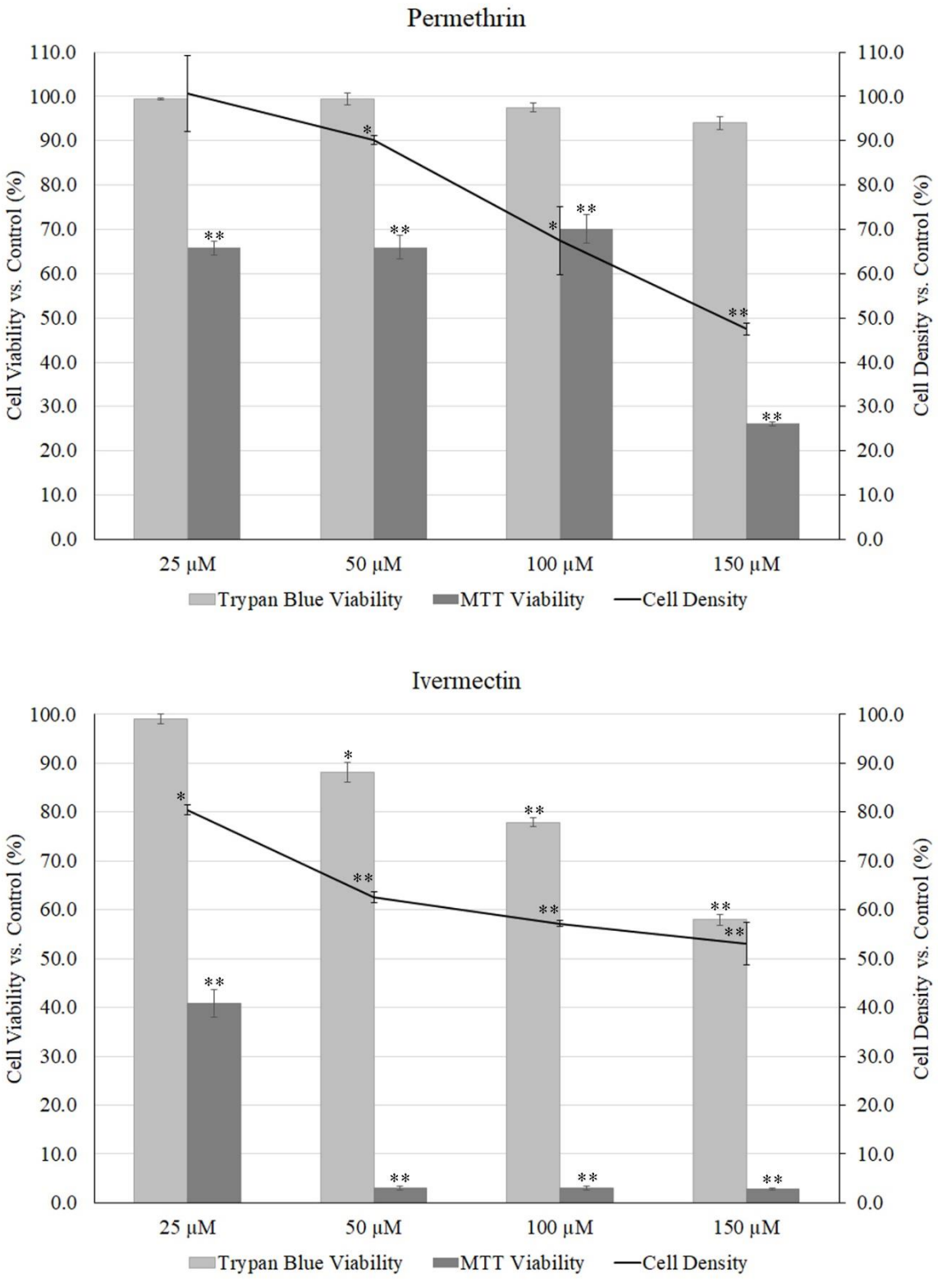


*Figure 37. IRE/CTVM19 cell viability with Trypan blue (light gray bars), MTT assays (dark gray bars), and cell density (black line) following treatment with increasing doses of permethrin (top) and ivermectin.(bottom) Data were reported as the mean of three independent experiments, with four replicates each, expressed as percentage of the control (0.5% DMSO) ± SEM. *p<0.05; **p<0.01*

*Table 8. Comparison table for results deriving from Trypan Blue exclusion and MTT assay. Data were reported as the mean of three independent experiments, with four replicates each, and expressed as percentage of the control (0.5% DMSO) ± SEM. *p<0.05; **p<0.01*

		Trypan Blue 0.4% w/v	MTT
DMSO	**0.5%**	96.1 ± 1.8	94.4 ± 1.6
Amitraz	**25 µM**	99.6 ± 1.1	92.3 ± 2.7
	50 µM	97.7 ± 0.9	96.2 ± 3.0
	100 µM	97.9 ± 0.3	91.9 ± 1.8
	150 µM	92.4 ± 0.8	69.4 ± 1.4**
Fipronil	**25 µM**	92.6 ± 0.4	70.8 ± 1.4**
	50 µM	90.4 ± 0.7	53.3 ± 2.7**
	100 µM	85.2 ± 1.4*	15.8 ± 0.9**
	150 µM	60.8 ± 1.6**	6.6 ± 0.4**
Permethrin	**25 µM**	99.4 ± 0.2	65.8 ± 1.6**
	50 µM	99.5 ± 1.4	65.9 ± 2.7**
	100 µM	97.5 ± 1.0	70.1 ± 3.3**
	150 µM	94.0 ± 1.4	26.1 ± 0.5**
Ivermectin	**25 µM**	99.1 ± 1.1	40.8 ± 2.9**
	50 µM	88.1 ± 2.0*	3.0 ± 0.3**
	100 µM	77.9 ± 0.9**	3.0 ± 0.4**
	150 µM	58.0 ± 1.1**	2.8 ± 0.3**

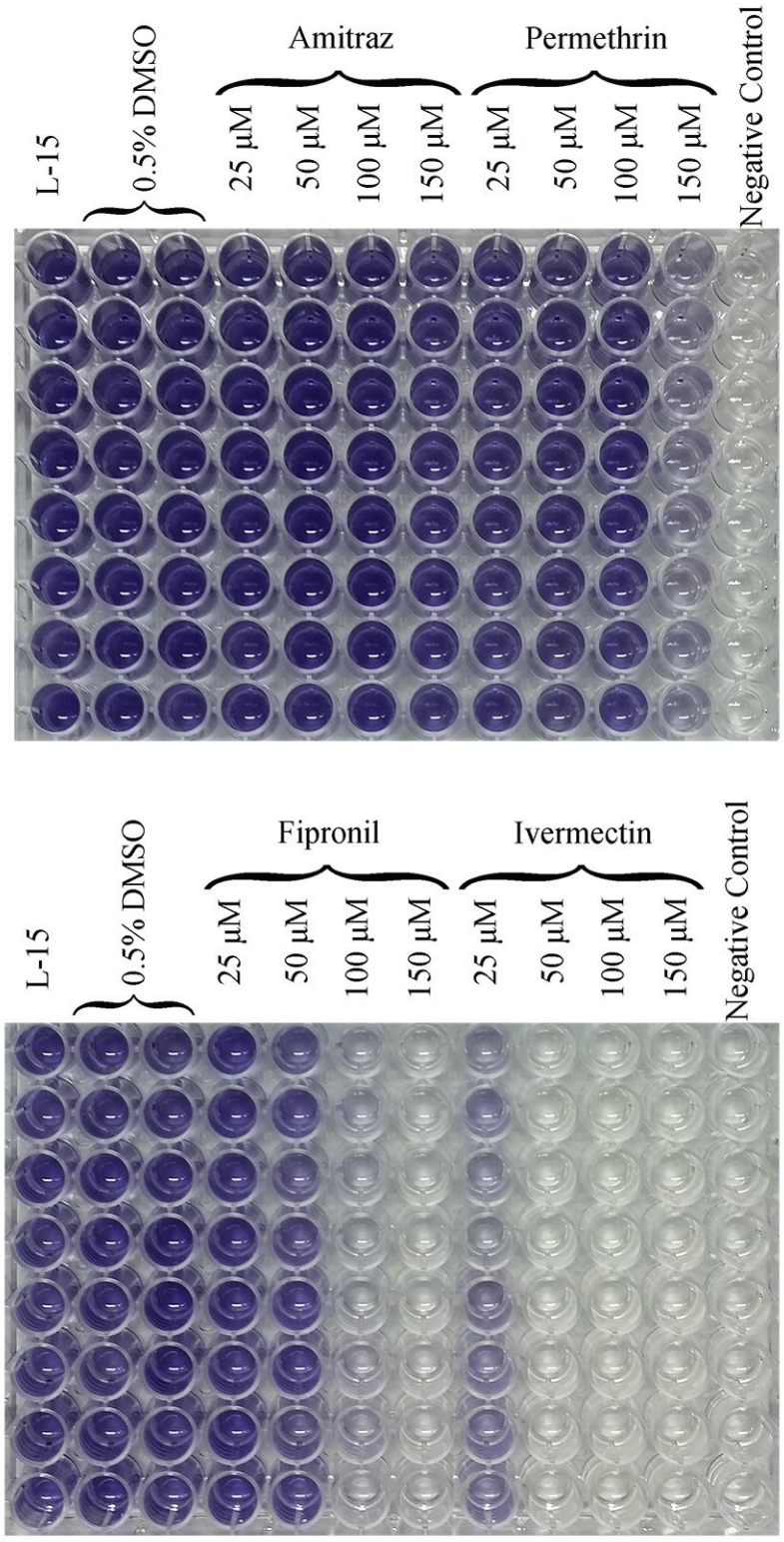


Figure 38. Example of MTT assay on IRE/CTVM19 cell line. Increasing concentrations of amitraz did not induce macroscopic alterations in tetrazolium reduction. Permethrin had a negative effect only at the highest concentration (150 µM). Fipronil induced a progressive reduction in formazan formation, while ivermectin had negative effects starting from 25 µM.

ABCB gene expression

Figure 39 and 40 show real-time PCR results following acaracide treatments. ABCB genes showed different modulation patterns. Contrary to first treatments with ivermectin, *ABCB1* gene expression was detectable, and gave interesting results because it showed a dose-dependent down-regulation with all molecules included in this study. *ABCB1* modulation was always strongly significant at the highest concentrations (100 µM and 150 µM, $p<0.01$), except for amitraz 100 µM, where p-value was less than 0.05. Fipronil and ivermectin were able to reduce *ABCB1* expression even at lower concentrations (FIP 25 µM and 50 µM, and IVM 50 µM, $p<0.05$).

Amitraz treatment did not show any other significant modulations of ABCB genes, except for a slight up-regulation of the *ABCB10* gene expression at 50 µM ($p<0.05$).

Fipronil treatment showed even a strong dose-dependent up-regulation of *ABCB6* up to 100 µM ($p<0.001$), but had no effect at 150 µM. Moreover, fipronil allowed the up-regulation of the *ABCB8* gene at 100 µM ($p<0.01$) and a strong increase of the *ABCB10* gene expression at 150 µM (+136% compared to control, $p<0.001$).

Permethrin treatment induced significant up-regulation in the expression of *ABCB6*, *ABCB8* and *ABCB10* genes at 150 µM ($p<0.01$).

Finally, ivermectin treatment showed a down-regulation of all the genes under investigation. The modulation could be related to dose-dependent patterns, above all in the expression of *ABCB6* (with the exception of IVM 150 µM) and *ABCB8*, with p-values less than 0.01. The highest concentration of ivermectin induced even a significant down-regulation of the *ABCB10* gene expression ($p<0.05$).

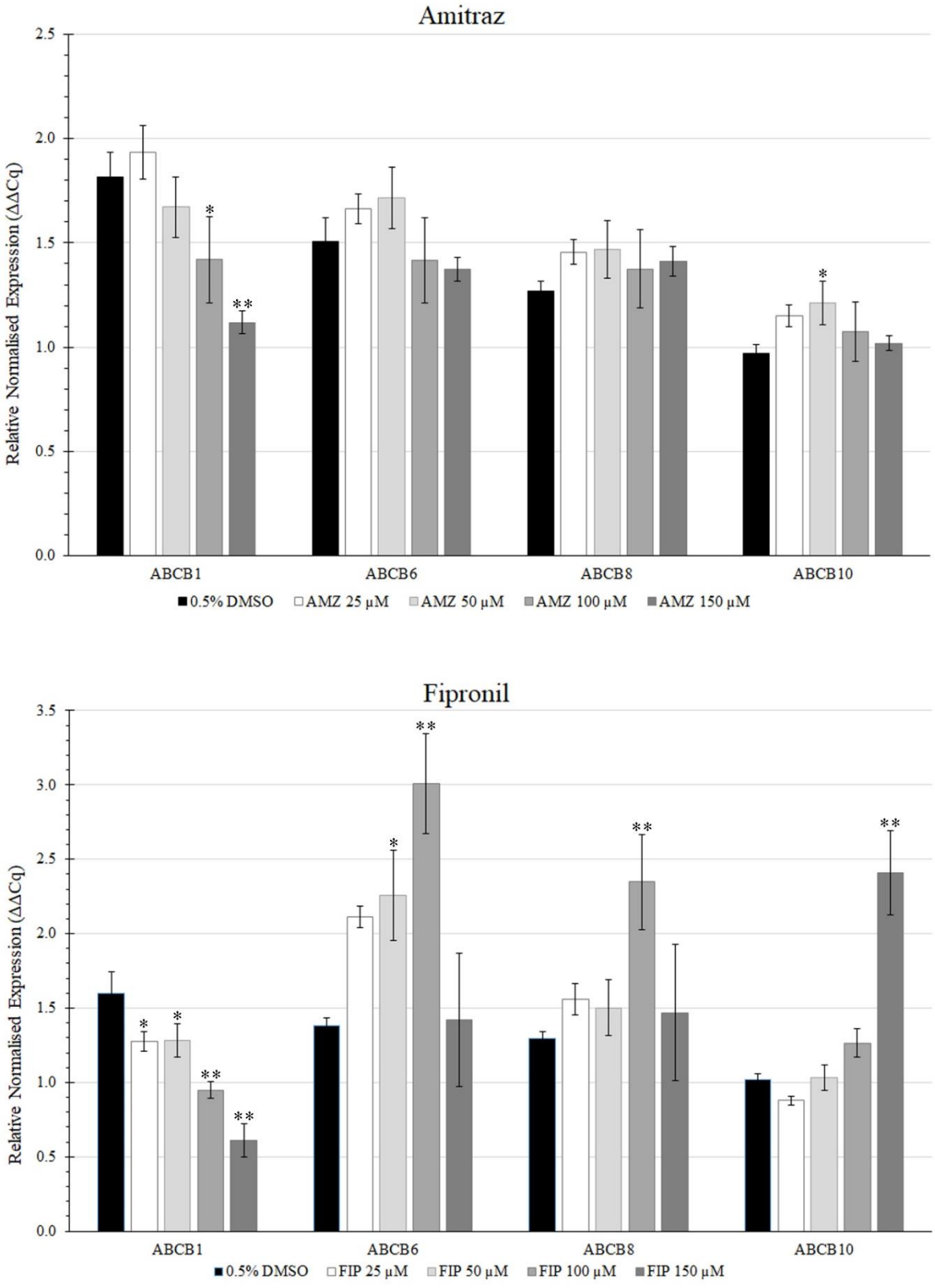


Figure 39. Expression of ABCB1, ABCB6, ABCB8 *and* ABCB10 *genes in IRE/CTVM19 cells treated with amitraz (top) and fipronil (bottom) at increasing concentrations. Data were reported as the mean of three independent experiments, with four replicates each, expressed as relative normalised expression (ΔΔCq) vs. time zero. *p<0.05; **p<0.01*

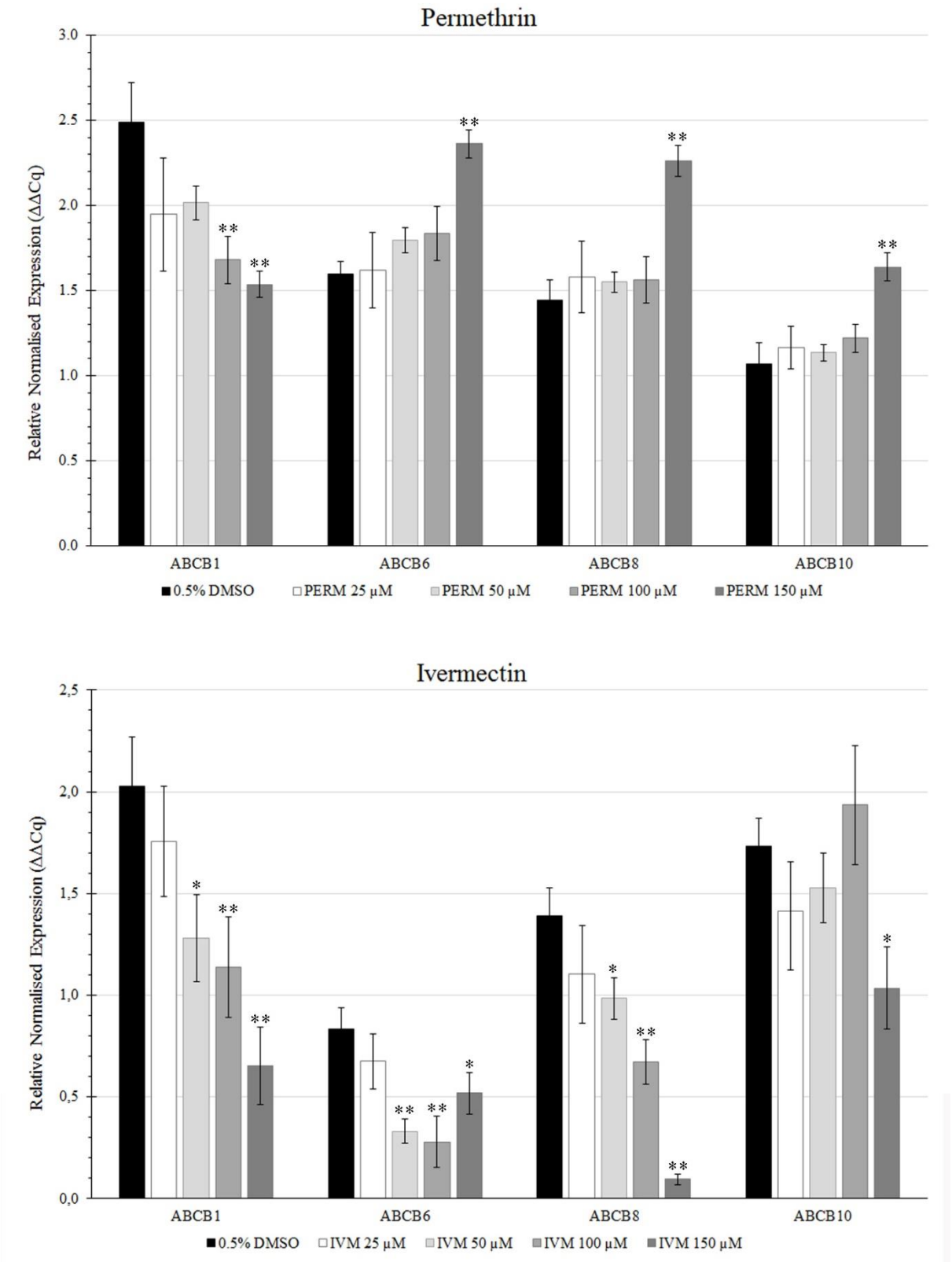


Figure 40. Expression of ABCB1, ABCB6, ABCB8 *and* ABCB10 *genes in IRE/CTVM19 cells treated with permethrin (top) and ivermectin (bottom) at increasing concentrations. Data were reported as the mean of three independent experiments, with four replicates each, expressed as relative normalised expression (ΔΔCq) vs. time zero. *p<0.05; **p<0.01*

IRE/CTVM19 mRNA analysis

The twelve raw data files (two for each sequenced sample) were of good quality, with sequence lengths ranging between 98 and 101 bp. However, the *FastQC* analyses reported the presence of sequences with high duplication levels, likely due to the presence of rRNA (removed through the *SortMeRNA* program) and of adapters used for the preparation of the libraries (removed through the *Trimmomatic* program).

The output of *Trinity* program was a .fasta file, which groups transcripts into clusters based on shared sequence content. A total of 435326 transcripts were clustered, and following filtering at 2.5 FPKM resulted into 31097 final transcripts.

Preliminary annotation of these transcripts led to the identification of several ABC transporters, belonging to seven families, from A to G, as previously reported by others. However, proteins homologous to the ABCB1 transporter could not be identified, nor proteins belonging to the H subfamily, which are arthropod-specific. Moreover, preliminary analyses of differential gene expression between control vs. treatment conditions did not generate significant data on the modulation of the ABC gene expression.

A

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Figure 41. Sequences of putative ABCB genes deriving from transcriptome analysis. In particular of ABCB6 *(A),* ABCB8 *(B), and* ABCB10 *(C).*

5. Discussions and Conclusions

Discussions

Ticks are arthropods of great veterinary interest and are the second most important vector of human and animal disease, after mosquitoes. Increasing attentions to these ectoparasites are attributed to their constant expansion throughout higher latitudes and altitudes, as well as to their establishment in new environments [9, 11, 14, 64, 65].

The continuing spread and diffusion of ticks is determined by numerous factors. First of all the climatic changes we are witnessing in recent decades. The rise in global temperatures, resulting in the absence of cold winters, allows the maintenance of ideal conditions for tick survival and development [11].

However, this is not the only reason. Humans are also playing a fundamental role in this problem. In fact, many studies conducted so far have been able to establish an indissoluble link between human activities and the spread of these arthropods [13]. In fact, the natural hosts of *Ixodes ricinus* (consisting of small, medium and large wild vertebrates) are changing their behaviour, as a result of agricultural policies, creation of green and/or protected areas in urban or peri-urban areas, deforestation and reforestation. The consequence is a sudden appearance in inhabited areas, with high probability of tick spreading. This is followed by a greater chance of contact with humans and animals, resulting in greater risk of infection with transmitted pathogens. In fact, the increasing cases of tick-borne encephalitis (TBE) in Scandinavia or in Eastern Europe is not a coincidence [72, 78].

Therefore, in this context of increasing risk for public health, guidelines for treatment and prevention of tick infestations are particularly important. As shown in Chapter 3, different classes of drugs are available on the market for tick and ectoparasite control. Ivermectin, amitraz, fipronil and permethrin are just some of the molecules belonging to the most important classes of drugs, and together constitute a large part of the market.

However, the uncontrolled use of these ectoparasiticides, also used in agriculture, is leading to phenomena of drug resistance, as happened in the past with carbamates and organophosphates [16-20, 179].

The molecular mechanisms at the base of acaricide resistance have not yet been fully clarified, but ABC transporters likely play a key role. They are active carriers expressed on the membranes of different cellular compartments and they are able to direct molecules counter-gradient due to the energy freed by the hydrolysis of two ATP molecules [272-274]. The peculiarity of these carriers is the low specificity for the molecules that they are able to recognise and their involvement was initially correlated with drug resistance to chemotherapy in oncologic patients. However, genomic and proteomic studies have shown how these transporters are highly conserved in eukaryotes and prokaryotes, and highlight their primary role in cellular detoxification processes [275-277]. Starting from these initial studies, a new current of research opened, focusing on evaluation of the involvement of these transporters in the mechanisms of drug resistance in arthropods. The results were surprising, with several publications that were able to show an increase in the gene expression of ABC transporters in pesticide-resistant arthropods [28-30, 268-270, 290, 295, 311, 332, 346].

For a better comprehension of the role and the mechanism of action of these proteins, it would be appropriate to study the *in vivo* models. Unfortunately, this is not always possible because of clear ethical and economic reasons. The maintenance of the biological cycle of ticks requires either feeding on mammalian hosts or with artificial membranes. These methods are expensive and ethically questionable. This is particularly true for the three-host tick *I. ricinus,* the most widespread tick species in Italy and Europe. In optimal conditions of temperature and relative humidity, the biological cycle of this species has an average life of about 2-3 years, and it may be longer in nature. In this context, the research of an alternative *in vitro* model, which is the main objective of this project, becomes of paramount importance.

In order to obtain preliminary validation of the model, several analyses were carried out. The first involved the determination of the time of cell duplication to evaluate the appropriate time-point for subsequent pharmacological treatments. The preliminary test estimated a doubling-time of about 10 days, particularly long for a continuous cell culture, especially of embryonic origin, but in line with other cellular lines, derived from different species [34]. The data could be justified simply by taking into consideration the slow biological cycle of *I. ricinus*.

Since this is the first study that has evaluated the behaviour of IRE/CTVM19 culture following drug treatments, the first problem to solve was the choice of the concentrations of molecules that had to be used. The first drug on which we focused our attention belongs to the class of macrocyclic lactones, the most marketed and effective antiparasitic drug class for helminths and ectoparasites, such as ticks and mites. Introduced to the market in the early 1980s, it has been remarkably successful in antiparasitic treatment. It acts at the level of glutamate-gated

chloride channels, mainly expressed at the central nervous system level, with a high selectivity for invertebrates [194, 195].

The inclusion of ivermectin in the study was also the result of widespread literature search, according to which macrocyclic lactones represent an excellent substrate for P glycoproteins (*mdr-1* genes, transporters of the subfamily B) [284]. Based on a previous *in vitro* study on continuous cell cultures of *Rhipicephalus microplus* (BME26), that hypothesised the involvement of ABC transporters in drug resistance mechanisms in ticks, the first concentrations of ivermectin for treatment were selected, trying to increase the upper limit of analysis, due to the slow metabolism of the culture under examination [34].

Changes observed in cellular morphology have not been accompanied by alterations in viability percentages. Considering the results achieved by Pohl *et al.* on *Rhipicephalus microplus* BME26 line, IRE/CTVM19 data appear to be very interesting, as the line is able to survive at higher concentrations of drug without any sort of selection. In fact, the highest concentration tested by Pohl was 9 μg/ml on a culture selected after 46 weeks of treatment at increasing concentrations of ivermectin, while the IRE/CTVM19 culture is able to admirably tolerate a concentration of about 30 μg/ml (33 μM) for 10 days. In addition, other contrasts with the same work comes from the expression analysis of ABC transporters, different for both studies except for the *ABCB10* gene [34].

Although *RmABCB10* gene expression in the study of Pohl *et al.* increased by 7.5 times compared to the control, IRE/CTVM19 did not show any significant modulation of the same gene, as no other tested gene had any significant alterations to its expression pattern, excluding the *ABCB8* gene after the treatment with ivermectin 22 μM. These preliminary results have led to the hypothesis of the presence of parallel detoxification mechanisms, different for the two tick species.

For example, in the same study by Pohl *et al*. it is clear that, after ivermectin treatment, *RmABCC1* and *RmABCB7* genes are involved in following cellular detoxification processes, although they have used lower drug concentrations than those used in this work. Unfortunately, in the first phase of the project it was not possible to detect the presence of the *ABCB1* gene, probably because of RT-PCR reaction-setting errors with the instrument used.

The most interesting results were surely achieved in the second part of the project, following amitraz, fipronil and permethrin treatment, as there are no previous literature studies on the use of such molecules in tick cell lines. Treatment with higher concentrations of ivermectin also generated very useful data for understanding the detoxification processes in the cell line under investigation.

As reported in the results, the four molecules, tested in the range 25-150 μM, have led to significant changes in cellular morphology, especially at higher concentrations. As described above, continuous cell cultures are constituted by two or more cell types that, of course, may respond differently to pharmacological treatments. In particular, the cytopathic effects common to all molecules were attributable to the presence of different cellular populations. The first was subjected to enlargement and vacuolization of the cytoplasm, while the second sub-population was characterized by typical elements of apoptotic cells, as characterized by a more compact form, an evident alteration of the condensation state of the chromatin and the integrity of the nuclear membrane, as shown in the Figure 34.

This type of alteration was not entirely unexpected, since several studies have reported that the cytopathic effects of these acaricides are due to the induction of the synthesis of oxygen (ROS) and nitrogen (RNA) reactive species. Oxidative stress is one of the major causes of cellular damage associated with the use of these molecules, accompanied by neurotoxicity and inflammatory stimuli, which can alter the homeostasis of macromolecules, such as lipids, proteins and DNA, resulting in the induction of apoptosis [258-260].

In this context, data on cell viability, measured by two different tests (i.e. Trypan Blue exclusion test and MTT assay), was very interesting. The exclusion test did not show any differences in viability after amitraz and permethrin treatment, even at higher concentrations. Conversely, treatments with fipronil and ivermectin showed a dose-response progression, with statistically significant values starting from 100 μM for fipronil and 50 μM for ivermectin. In addition, the lowest dose of the latter (25 μM) confirmed the data on cell viability generated in the first part of the project.

The MTT test, on the other hand, confirmed viability data only in the amitraz treatment and only up to a concentration of 100 μM. At the highest concentration and in the rest of the treatments, strongly contrasting values were found, especially in the case of ivermectin, where at 25 μM the cell viability dropped to 40.8% and finally collapsed to 3% at 50 μM (Table 8). It seems fascinating, however, the negative progression after treatment with fipronil, which followed the constant decrease determined by the Trypan Blue analysis. The possible explanation has to be searched in the bases of the two examined assays.

The exclusion test with Trypan Blue uses a dye that is not able to cross the cell membrane of still-living cells, but it can permeate through the cytoplasm of death cells, or cells on the verge of dying. Therefore, the major limitation of this kind of assay is the inability to discriminate between healthy cells and live cells that have lost their functionality, as reported in some of the

studies carried out on *Spodoptera frugiperda* cell lines treated with permethrin and *D. melanogaster* cell lines treated with fipronil [347, 348].

Conversely, the MTT assay is a colorimetric assay, based on the reduction of tetrazole (yellow) to formazan salt, an insoluble dark violet crystal, mediated by mitochondrial dehydrogenases. It is definitely the most used test to determine cell viability, especially following pharmacological treatments, as in this project, and contributed to the production of numerous scientific publications. However, this test also has several limitations. The first one is given by the cell culture medium. In fact, according to the data collected by Vistica *et al.* in 1991, several culture media are not recommended for the use of MTT assays, since they lack glucose, as in the Leibovitz's L-15. The lack of this sugar leads to a drastic decrease in the amount of cellular NADH (reduced form of the nicotinamide adenine dinucleotide coenzyme, present in all living cells), resulting in a reduction of cellular metabolism levels [349]. In the present study, however, at the end of the incubation time (3 hours), the controls had a very strong purple colour; symptom of the presence of large amounts of formazan salts (Figure 38). Secondly, and as previously mentioned, the reduction of tetrazole salt occurs in cellular mitochondria, therefore MTT assay evaluates mitochondrial activity and, hence, an evaluation of cellular metabolism. For example, it does not take into account the possibility that these cells may block their own metabolic processes following the presence of an unfavourable growth environment (process called diapause), such as in *in vivo* ticks, from which they are derived. This mechanism could therefore explain the discordant results obtained through these two methods. Cells exposed to pharmacological treatments may undergo a "forced" quiescent state: in this way, the metabolically active cells are drastically reduced (low MTT values), but remain alive (low Trypan Blue values). Any lowering of cellular viability values measured through Trypan Blue exclusion assays, on the other hand, as observed here in cells treated with high drug concentrations, may be due the inability of the cells to enter into a quiescent phase, thus leading to cell death.

These considerations could also partly explain the results of the analysis of the expression of the ABC genes under investigation. All drugs, in fact, induce a progressive decrease in expression levels of the *ABCB1* gene, the only transporter among those studied to be located on the cell membrane. A progressive shutdown of cellular metabolism levels also leads to fewer transcripts for the *ABCB1* gene. However, this cannot be the only explanation of the obtained results, as the drugs have a contrasting effect on the other ABC transporters taken into account, all expressed in mitochondria. In particular, it is curious to observe how fipronil induced a dose-dependent response in the expression of the *ABCB6* gene up to a concentration of 100 μM, then

return to baseline levels at 150 μM. As well as the molecule induced statistically significant increase of the expression of the *ABCB8* gene only at 100 μM, and of the *ABCB10* gene only at the highest concentration (expression more than double compared to controls).

Treatment with permethrin, which had no influence on cell viability measured through Trypan Blue, and a constant reduction of 65-70% with MTT in the first three concentrations used, induced a positive stimulation in the expression of the three genes (*ABCB6*, *ABCB8*, and *ABCB10*) only and exclusively at 150 μM. To that concentration, the percentage of viability cells, measured through MTT assay, was only the 26%.

Ivermectin, on the other hand, leads to a reduction in expression levels of all the genes investigated, especially at the highest concentration. This is particularly evident in *ABCB10* gene expression, while in *ABCB6* and *ABCB8* genes there is a kind of dose-response (with the exception of *ABCB6* at 150 μM). The decreased expression of the *ABCB10* gene contrasts with the data reported on other studies carried out on two different *R. microplus* cell lines, BME6 and BME/CTVM6, where the same gene appeared to play an important role in drug resistance [33, 34]. This conclusion was particularly evident in the second cell line, which was derived from a field tick resistant to ectoparasiticides [33].

All of these data, taken together, suggest the presence of other proteins, both associated to cellular membrane, organelles and other compartments (such as mitochondria and hemosomes) involved in the ejection or extrusion mechanisms of exogenous molecules, as well as the induction of enzymes involved in their metabolism.

Detailed analysis of the transcript of the line treated with fipronil 100 μM could clarify, at least partially, these doubts. Preliminary transcriptome analysis showed the presence of several ABC transporters, belonging to seven families, from A to G, as reported in several publications. However, proteins homologous to the ABCB1 transporter could not be identified, nor proteins belonging to the H subfamily, which are arthropod-specific. This might be due to the high FPKM cut-off, which excluded selected transcripts from the annotated list. Even the absence of significant values on the modulation of the *ABC* gene expression might be due to the stringent conditions of C (log fold change) and P (p-value cut-off) values used for differential expression analyses. Follow-up analyses are currently underway to help elucidate this point.

Conclusions

This is the first study conducted on an *Ixodes ricinus* cell line following pharmacological treatment with some of the most commonly used drugs to control ticks. Ivermectin, amitraz, fipronil and permethrin belong to relatively recent classes of active principles. Drugs based on these molecules have been introduced onto the market over the last decades and are widely used as pesticides, even to overcome the phenomena of drug resistance to traditional molecules (such as carbamates and organophosphates). Their use has increased over time to control ticks, lice and mites in farm animals, companion animals and the environment. Although they have different mechanisms of action, all appear to be specifically selective for the arthropod nervous system. However, continuous exposure to these molecules could lead to the increasing appearance of resistant parasites, as described in the literature. Therefore, the development of a continuous cell line for the study of these phenomena is absolutely necessary.

The maintenance of the IRE/CTVM19 cell culture is very simple and requires few small steps, such as growth in flat-side tubes, no CO_2 and, above all, high-density cultures (cell seeding at much less than 10^6 cells/ml result in viable cell cultures, but characterized by strongly vacuous cells with irregular appearance). The most important feature, which makes this cell culture the ideal candidate for the development of a new *in vitro* model, capable of definitively replacing the *in vivo* research, is the heterogeneity of the cell type of which it is constituted. As described above, embryonic cell lines are characterized by different cell types, potentially able to represent and mimic the behaviour of all tick tissues. However, this great advantage is also its biggest disadvantage, as it is reported that cellular composition can differ enormously even within sub-cultures deriving from the same tube [32, 242].

Furthermore, in the search for drug resistance mechanisms and implications of ABC transporters, it is still not known whether the expression of these genes undergoes variable temporal dynamics, as reported in other arthropods, such as *Anopheles stephensi* [290, 311]. In fact, as previously mentioned, a common hypothesis is that different ABC transporters may have different degrees of relevance in detoxification during developmental stages of ticks.

Moreover, it has never been verified whether the tick from which this line was derived was already resistant to some of the ectoparasiticides used in the veterinary field, with possible highest basal expression levels of the ABC transporters.

Therefore, the first step has been completed to include IRE/CTVM19 cell line among the useful tools for the study of the molecular mechanisms beyond drug resistance phenomena in ticks, and new insights may be obtained following the completion of the transcriptome analysis of

cell culture. Moreover, this tick cell line might provide an optimal substrate for new molecules, or compounds, that have to be tested. However, further studies have to be carried out to assess the role of the different ABC transporters in response to the numerous classes of drugs currently in the market for the control of tick infestations.

Acknowledgements

My greatest gratitude to Prof. Laura Kramer, who gave me the opportunity to discover the fantastic and interesting world of ticks, and parasites in general. Thanks for her limitless courtesy and patience, and her continuing support during the entire PhD course.

Heartfelt thanks to Prof. Marco Genchi and Dr. Chiara Cattabiani for the incessant assistance in the Parasitology lab, for each day spent in our office and for the mountain of coffee we took all together. Thanks for every given suggestion and for the sincere friendship we have matured during these three years.

Many thanks to Dr. Lesley Bell-Sakyi, who provides IRE/CTVM19 cell cultures. Thanks for her kindness and cordiality during my stay at the Pirbright Institute.

Many thanks to Dr. Cinzia Cantacessi, who gave me the chance to learn more about bioinformatics. Thanks for her endless willingness, hospitality, and continual advices during my stay at the University of Cambridge.

Finally, thanks to everybody who, one way or the other, contributed to the development of this PhD project.

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