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Biological and Biochemical Bases of Pesticides Resistance in *Rhipicephalus* (*Boophilus*) *microplus*

Rodrigo Rosario-Cruz and Delia Inés Domínguez-García

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Abstract

Several arthropod species are important vectors of pathogens that cause disease in humans, animals, and plants, including protozoa, nematodes, bacteria, and viruses. Arthropods are also pests competing with humans for food and parasitize farm animals, decreasing their productivity. Historically, arthropod pests and disease vectors affecting public health, crop yields, and livestock production have been managed through the intensive use of pesticides. The widespread use of pesticides is a major problem because most of the economically important arthropod species have developed resistance to currently used pesticides. The impact of pesticide resistance is multifactorial and involves losses due to the heavy use of pesticides, environmental pollution, decreased profitability, food contamination, and public health problems due to pesticide exposure. An indirect consequence of pesticide resistance is the mortality caused by arthropod-borne diseases such as dengue and malaria in humans and babesiosis and anaplasmosis in cattle. The understanding of molecular mechanisms and adaptations to resistance in arthropods is an important issue. However, the molecular mechanisms of pesticide resistance remain to be fully understood. Understanding of resistance mechanisms will contribute significantly to improve integrated managements programs and to discover new targets for vaccine development to mitigate the effects of pesticide-resistant arthropods on agriculture and public health.

Keywords: Pesticides, resistance, pests, vectors, mechanisms of resistance, insecticide resistance

1. Introduction

Insects, ticks, and mites are the main groups of arthropods, including species that can be pests and disease vectors. Historically, the problem with arthropod pests and disease vectors affecting public health, crop yields, and livestock production has been managed through the

massive use of pesticides. The widespread use of pesticides exerted strong selective pressure and now several of the most economically important arthropod species are resistant to currently used pesticides.

Pesticide resistance in arthropod species of public health, agricultural, and veterinary importance has become a major problem. This situation presents a threat for societies around the world because several arthropod species attack crops and thus compete directly for food with humans, and other species are important vectors of infectious agents causing diseases in humans, livestock, wildlife, and plants [1]. The origin of resistance arises through evolutionary genetic changes, causing modifications in the molecular structure of the target site or promoting changes in multigenic metabolizing enzyme systems, resulting in high hydrolysis rates or sequestration of pesticides as well as a reduced capability to penetrate the outer chitin protective layer.

Here we review the known molecular and biochemical mechanisms of pesticide resistance and do a gap analysis of processes involved in the evolution of insensitivity to pesticides among arthropods that remain to be fully understood. Significant advances have been achieved in our knowledge of the processes involved in resistance to pyrethroids. More research is required to decrease the knowledge gaps regarding mechanisms of resistance to organophosphates (OP). Less is still known about resistance to amitraz, macrocyclic lactones, and fipronil, whose mechanisms of action appear to involve complex and multifactorial evolutionary mechanisms.

An example of the problem with pesticide-resistant pests is the cattle tick *Rhipicephalus (Boophilus) microplus*, which is regarded as the most economically important ectoparasite of livestock globally and ranks sixth among the most pesticide resistant pests globally. This cattle tick affects animal health and production in tropical and subtropical regions of the world directly through its obligate hematophagous habit and indirectly by serving as vector of pathogens like *Babesia bovis*, *B. bigemina* and *Anaplasma marginale*, which cause the deadly bovine diseases babesiosis and anaplasmosis, [1, 2]. The control of cattle ticks, bovine babesiosis and anaplasmosisis a costly problem that prevents the livestock industry in Latin America and other parts of the world achieving its full potential [1]. The almost complete reliance on pesticides to control cattle ticks and associated diseases has been a strong selective force for tick populations that now are resistant to multiple classes of acaricides [3]. These developments highlight the super genetic plasticity and exquisite adaptability of arthropod pests and vectors, which has enabled them to become resistant to most classes of synthetic pesticides humans have discovered and developed for commercialization.

2. Resistance, adaptation and coevolution

Resistance to synthetic pesticides is a genetic condition that confers an arthropod population the capability to get adapted to a toxic environment through a selection process driven by human activity [4]. Based on this concept, the phenomenon of pesticide resistance can be understood as an important model based on natural selection processes [5]. Considering pesticide resistance as a biological model facilitates the study of evolutionary adaptations by

arthropods living under selection pressure through constant exposure to pesticides used by humans [6]. However, the natural history of pest-host interactions in a way preadapted arthropods to become resistant to synthetic pesticides.

As a naturally occurring process, the coevolution between plants and arthropods enabled biological and chemical interactions over hundreds of millions of years [7]. As a result of the interactions between these two groups of organisms [8], arthropod species conserved within their genomes genes conferring an evolutionary advantage, or traits that have allowed them to survive. These types of interactions called allelochemical interactions have allowed plants and arthropods to develop very sophisticated molecular mechanisms to maximize their chances of survival. Plants evolved to be a factory of potent allelochemicals such as natural poisons, toxins, and repellents that function as a natural mechanism of defense against predators. As an example of coevolution, herbivore arthropods adapted by evolving a machinery of metabolic hydrolyzing enzymes to prevent poisoning by allelochemicals acquired during plant feeding [9–11]. This resulted in what could be described as natural resistance in arthropods selected through exposure to plant allelochemicals, which took millions of years. Arthropods were in a way preadapted to become resistant to synthetic pesticides rapidly in terms of evolutionary time [12]. Another predisposing factor for this rapid adaptation is the structural analogy between secondary metabolites produced by plants and synthetic pesticides designed by humans to control pests [13, 14].

The development of resistance to chemicals depends on the evolutionary forces exerting selection pressure and the consequent adaptive processes involving selection of genetic variations caused by random mutations, or genetic rearrangements resulting from exposure to pesticides [7], which is reflected in the selection and reproduction of individuals with resistant phenotypes capable of surviving pesticide concentrations that are lethal to the wild-type populations.

Resistance occurs when the selection of low frequency naturally occurring mutations within the genetic pool of a population allows a small fraction of individuals to survive the toxic effect of compounds used as pesticides [15]. Prior to initial exposure of the population to pesticides, there are few individuals with resistant genotypes and most of them are susceptible. When a pesticide is applied, those few survivors have an adaptive advantage. Therefore, they survive initial exposure and reproduce, and the frequency of resistant genotypes in the progeny increases. If repetitive and sustained applications of the pesticide on the population occur, the susceptible individuals are eliminated, which drives the selection of resistant genotypes in the population. At this point, the diverse genetic traits conferring an advantage to survive pesticide exposure are transferred to the progeny and the efficacy of the pesticides decreases.

3. Molecular basis of pesticide resistance

In general, acaricide resistance can be the result of increased metabolic detoxication or target site modification [4]. Metabolic detoxication is frequently the result of increased enzymatic activity by isozymes encoded by multigene families such as cytochrome P450, glutathione-S-

transferase, and carboxylesterase [16, 17]; all these enzymes hydrolyze or sequester different kinds of pesticides. Exposure to a pesticide can exert enough pressure to select an enzymatic system or a specific isozyme within each family. Esterase isozyme overexpression is generally accepted as a mechanism involved in OP resistance. However, in the Coatzacoalcos laboratory strain of *R. microplus*, designated as such to reflect the name of the village in Mexico where the original tick population sample was obtained, metabolic detoxication has been identified by its efficient esterase activity resulting from enzyme overexpression as a resistance mechanism for permethrin that belongs to the pyrethroid chemical class of pesticides. This strain has been toxicologically characterized using the larval packet test (LPT) [18], which helped to elucidate the esterase-based mechanism of resistance to permethrin. The *R. microplus* Coatzacoalcos strain exhibits a significant enhanced capacity to hydrolyze permethrin as well as an increased esterase activity. This suggests an esterase based metabolic mechanism as a main component of permethrin resistance [19]. The esterase gene responsible for permethrin resistance was identified and named *CzEST9*. It is also known that the overexpression mechanism of this isozyme is the result of *CzEST9* duplication in the Coatzacoalcos strain that leads to metabolic detoxication through the overexpression of esterase 9 activity in *R. microplus* [20]. The sequence of *CzEST9* gene has been determined and the recombinant product yielded a 62.8 kDa protein [19]. Since the Coatzacoalcos strain does not include the *Kdr* variation in the sodium channel gene found in other Mexican strains of *R. microplus*, it is suggested that there are two independent mechanisms of acaricide resistance to pyrethroids. However, common mechanisms of acaricide resistance to pyrethroids in Mexico apparently involve the presence of sequence variation in the sodium channel gene [21].

The sodium channel is the known target site of pyrethroids. Sequence variation in the sodium channel prevents pyrethroids from attaching to the target site due to an alteration in the sodium channel stereochemical structure. For this reason, the process is described as target site modification mechanism, or *Kdr*-type resistance (Knock down resistance). This is one of the mechanisms of pesticide resistance in insects that is better understood.

Two important allele variants occurring in the sodium channel gene associated with pyrethroid resistance in the cattle tick *R. microplus* are the variation occurring in domain III segment 6 (III-S6) [22] and the variation occurring at the bridge joining segments 4–5 in domain II (II-S4-5) [23]. The former is a Phe-Ile substitution produced by a nucleotide variation at domain III-S6 that was first reported in Mexican tick strains. Its role and contribution to pyrethroid resistance has been confirmed [21]. The other is a Le-Ile substitution found thus far only in Australian tick strains; this variation is very similar to a variation found in the crop insect pest *Bemicia tabaci* [23].

Findings on the diversity of allele variants occurring in the sodium channel gene associated with pyrethroid resistance in the cattle tick *R. microplus* have been confirmed by experiments based on differences in melting temperatures (Tm) of sodium channel allele specific gene fragments obtained with single larvae DNA from México and Australia. These experiments revealed that substitution III-S6 (Phe-Ile) only occurs in Mexican tick populations whereas substitution II-S4-5 (Le-Ile) only occurs in Australian tick strains [24]. The information available suggests that there are at least two different and independent mechanisms involved based on

the different amino acid substitutions (Phe-Ile and Le-Ile) residing in different positions of the sodium channel protein sequence.

4. Biological basis of pesticides resistance

Allelochemical interactions are defensive processes or are involved in food competition mechanisms that different species employ to inhibit the action of natural enemies. Plant–arthropod coevolution is a natural selection mechanism driven by allelochemical interactions between plants and arthropods over millions of years [7]. As a result of the reciprocal inter-actions between these two groups of organisms [8], arthropods have conserved within their genomes all those traits conferring them the ability to inhibit or avoid toxicants produced by plants that function as defensive mechanisms against herbivorous insects [9, 10]. Sophisticated metabolic detoxication mechanisms have been developed by herbivorous arthropods in order to survive the exposure to toxic plant metabolites [11], which represents a natural process of resistance to plant toxicants. The preservation of these components in the genome of arthro-pods provides the foundation of molecular systems that allow them to get adapted and become resistant against pesticides currently used.

Insects affect the survival and reproduction of plants. Secondary metabolites like phenolic acids, flavonoids, terpenoids, steroids, alkaloids, and organic cyanides are produced by plants as part of defensive mechanisms. A coevolutionary race is established through these interac-tions where mutual selection pressure has led to the process of speciation to preserve natural equilibrium.

Adaptation to new environmental conditions requires the development of defensive processes through natural selection. Plants are biological engines producing a wide variety of natural defensive chemicals including repellents, antifeeding molecules, and poisons, some of which have been used as natural strategies to protect cultivars from plagues or to control vector-borne diseases. These mechanisms evolved separately in different herbivorous insects driven by diverse insect–plant interactions. In general, herbivorous insects feed on few plant species and plant species are attacked by pests specialized to overcome natural defensive substances. Herbivorous insects have developed the molecular machinery to metabolize most of the toxic material produced by plants, but not all toxicants are metabolized by all pest species.

Specialized insects have an adaptive advantage because their biochemical systems evolved to detoxify one or few potentially harmful substrates. The metabolic system of a polyphagous species reflects more diverse detoxication mechanisms against a wide variety of chemically defined plants. Thus, polyphagous insects have a "higher metabolic load". The activity of mixed function oxidases in the intestines of moths and butterfly larvae is higher in polypha-gous species than in species restricted to a single family of plants. Pyrethrins are part of the wide variety of allelochemicals metabolized by this family of enzymes [25].

5. Metabolic detoxication of pesticides

Plant–insect coevolutionary interactions drive species diversification and the set of genetic traits that allow pest species to survive exposure to a wide variety of secondary metabolites produced by plants. Such genetic traits evolved through evolutionary history, involving several highly specialized multigene families that are responsible for detoxication mechanisms of biotic and xenobiotic compounds. Examples of these multigene families include the glutathione transferases, mixed function oxidases, and carboxilesterases superfamilies [26]. These supergene families are capable of metabolizing a large amount of chemicals, some of which are currently used as pesticides.

Some mechanisms of resistance have been identified for several important arthropod vectors. Increased esterase activity is a major component of organophosphates resistance in *Culex* mosquitoes [27]. The enhancement of mix function oxidases also plays an important role in OP and pyrethroid resistance mechanisms [28], and the combination of mixed function oxidases and esterases in high concentrations has been detected in permethrin-resistant mosquitoes [29]. Resistance mechanisms in cockroaches include metabolic detoxication and *Kdr*-type resistance. However, detoxication mechanisms mediated by esterases and oxidases have been identified as the most frequent mechanisms of resistance [30].

6. Insensitive target site

Modifications of target sites as a result of point mutations on gene sequences have been also identified as mechanisms of resistance. Variations on genes encoding GABA receptors [31, 32], acetylcholinesterase [33], some detoxifying esterases [34, 35], and sodium channel gene sequences (22) have been discovered in different arthropods. The latter has been identified as *Kdr*-type resistance; this variation alters the molecular structure of the sodium channel, which is the target site of pyrethroid pesticides [36, 37].

Kdr-type resistance was firstly documented in *R. microplus* by He et al. [22]. It was shown that there is a variation in the sodium channel gene sequence at position 2134 where the base substitution of thymine by adenine (T2134A) results in an amino acid change from phenylalanine to isoleucine on the transmembrane segment 6 (S6), which is located on domain III of the para-type sodium channel gene [22].

Pyrethroid resistance in arthropods has been associated with nonsynonymous mutation on domains I, II, III, and IV of sodium channel genes [38, 39]. As already mentioned, two important variations have been previously identified in *R. microplus* sodium channel gene, a domain II variation (C190A) and the domain III variation (T2134A). The latter only found in ticks from Texas and Mexico [22, 40–42] and the former reported in Australia, Africa, and South America [23, 41, 42]. Although pyrethroid resistance in Mexican cattle tick populations has been mostly attributed to the domain III variation T2134A [22, 36, 39, 43], some authors have suggested that additional resistance mechanisms to the sodium channel variations must be present, since

genotype frequencies from screened populations do not account for the level of phenotypic resistance observed in field [22, 39, 41].

Previous studies have documented the occurrence of variations on an esterase gene associated with pyrethroid resistance; however, this phenomenon seems to be linked to a pyrethroid metabolic detoxication mechanism since it has been consistently found in pyrethroid-resistant ticks [35, 37]. These results suggest that pyrethroid resistance in ticks is the result of genetic traits involved in both metabolic and insensitive target mechanisms, depending on which gene the variation occurs.

7. Genomic perspectives for pest control

Applying the genomics approach to pesticide research offers the opportunity to advance our knowledge of the mechanisms of resistance and to find sustainable solutions to problems associated with pesticide resistance diagnosis, prediction, and prevention. This will also expand options to improve integrated pest management programs. In the case of livestock, a more rational use of pesticides could be achieved by combining genomics-based knowledge of acaricide resistance with the use of more efficient anti tick vaccines developed through modern technologies [43, 44].

The use of recombinant DNA technologies and the application of bioinformatics to mine genome databases such as GenBank, are powerful foundations to innovate diagnostic tools based on the identification and amplification of single nucleotide polymorphisms (SNPs) associated to target site insensitivity mechanisms [39, 42, 43]. Recent technologies such as polymerase chain reaction (PCR) is a powerful tool used to amplify or detect SNPs that can be employed as biomarkers of pesticide resistance, which provides an alternative to the time consuming bioassays that mitigates the risk of exposure to pesticides by laboratory personnel [21, 35–39, 44].

Genomics approaches are also enabling the design of new target antigens through *in silico* analysis of transcriptomic and genomic data to develop vaccines against ticks [43, 45], mosquitoes, other biting flies, and parasitic worms, as well as markers for pesticide resistance detection [46, 47]. The integration of molecular methods for pesticide resistance detection, prediction, and vaccine development efforts against hematophagous arthropods is an exciting alternative to manage the emergence of pesticide resistance and to improve vector and vector-borne disease control technologies.

8. Concluding remarks

Pesticide resistance is a preadaptive and genetic condition that implies the presence and selection of a collection of genes within a population of arthropods. A principal function of this suite of genes is to detoxify the chemicals used for pest control. Pesticide use is a strong selection force for resistance among pest populations.

Genetic plasticity in arthropods enables the emergence of resistance to currently used pesticides. Because of the natural host defense mechanisms evolved through millions of years, it is likely that arthropod pests are preadapted to become resistant to new pesticides, especially if the current intensive use of pesticides continues.

The growing problem of multiple resistance in arthropods all around the world demands research that can help us to better understand the mechanisms of pesticides resistance. This knowledge can be translated into improved diagnostic and predictive tools to mitigate the impact of pesticide resistance. Integrating genomics methods for pesticides resistance detection and vaccine development against hematophagous arthropods will improve strategies to prevent and predict the emergence of pesticides resistance that could lower the burden of pests and vector-borne diseases on humans, livestock, and wildlife.

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