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MOLECULAR TOOLS FOR DETECTION OF INSECTICIDE RESISTANCE

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ABSTRACT

Insecticide resistance is becoming problematic, day by day, to those who uses them invariably for the control of medical, veterinary and agricultural insect pests. In case of many insects, this problem is extending to almost all the major groups of insecticides. Since the first case of DDT resistance in 1947, the incidence of resistance has been increasing annually, at an alarming rate. It was estimated that there were at least 447 pesticide resistant arthropods species in the world today (Callaghan, 1991). Resistance to insecticides has also been developed by many insects to new insecticides with different modes of action from the main four groups. With the introduction of every insecticide class – cyclodienes, carbamates, organophosphates, pyrethroids, even biopesticides – cases of resistance surfaced within two to 20 years. Thus, the role of molecular tools in the detection of insect resistance is becoming increasingly important in the current area of uncontrolled insecticide usage, rapidly dropping lethal dosage levels to get quick understanding, of the status of resistance developed by the insects.

Key words: Insecticides, Resistance, Molecular Tools

Introduction

The development of resistance in the fields is influenced by various factors. These are biological, genetic and operational factors. Biological factors are generation time, number of offspring per generation and migration. Genetic factors are frequency and dominance of the resistance gene, fitness of resistance genotype and number of different resistance alleles. These factors cannot be influenced by man. However, such as treatment, persistence and insecticide chemistry, all of which may and therefore timing and dosage of insecticide application should be operational factors. Pesticide resistance is the adaptation of pest population targeted by a pesticide resulting in decreased susceptibility to that chemical. In other words, pests develop a resistance to a chemical through natural selection: the most resistant organisms are the ones to survive and pass on their genetic traits to their offspring (PBS, 2001).

Pesticide resistance is increasing in occurrence. In the 1940s, farmers in the USA lost 7% of their crops to pests, while since the 1980s, the percentage lost has increased to 13, even though more pesticides are being used (PBS, 2001). Over 500 species of pests have developed a resistance to a pesticide (Anonymous, 2007). Other sources estimate the number to be around 1000 species since 1945 (Miller, 2004).

Today, pests once major threats to human health and agriculture but that were brought under control by pesticides are on the rebound. Mosquitoes that are capable of transmitting malaria are now resistant to virtually all pesticides used against them. This problem is compounded because the organisms that cause malaria have also become resistant to drugs used to treat the disease in humans. Many populations of the corn earworm, which attacks many agricultural crops worldwide including cotton, tomatoes, tobacco and peanuts, are resistant to multiple pesticides (Berlinger, 1996).

Resistance can be defined an inherited ability to tolerate a dosage of insecticide that would be lethal to the majority of individuals in a normal wild populations of the same species. **Insecticide resistance** is apparent when a population stops responding or does not respond as well to applications of insecticides. In recent years, many of the resistance mechanisms have been detected and resistance detection methods have been developed. These mechanisms have divided into four categories: a) increased metabolism to non-toxic products, b) decreased target site sensitivity, c) decreased rates of insecticide penetration, d) increased rates of insecticide excretion. Mechanisms of insecticide resistance in insects are 1. Behavioral resistance 2. Penetration resistance 3. Metabolic resistance 4. Altered target-site resistance

Insecticide resistance detection techniques

The mode of action of the insecticides, duration life cycle, clutch size and availability of host determine rate of evolution of resistance. Documenting the dynamics of resistance plays another important role in the approach of its mitigation. Reliable, quick and effective techniques to distinguish between susceptible and resistant individuals are necessary (Gunning, 1993 and Brown, 1981).

1. Conventional Detection Methods: The standard method of detection in to take sample of insects from the field and rear them through to the next generations. Larvae or adults are tested for resistance by assessing their mortality after exposure to a range of doses of an insecticide. For susceptible and field populations, LD50 or LC50 values were calculated by using probit analysis.

2. Biochemical detection of insecticide resistance: Biochemical assays/techniques may be used to establish the mechanism involved in resistance. When a population is well characterized some of the biochemical assays can be used to measure changes in resistance gene frequencies in field populations under different selection pressure.

3. Immunological Detection Methods: This method is available only for specific elevated esterases in collaboration with laboratories that have access to the antiserum. There are no monoclonal antibodies, as yet, available for this purpose. An antiserum has been prepared against E4 carboxylesterase in the aphid *Myzus persicae*. An affinity purified 1gG fraction from this antiserum has been used in a simple immunoassay to discriminate between the three common resistant variants of *M. persicae* found in the UK field populations (Devonshire *et al*, 1996).

4. Detection of mono oxygenase (cytochrome P450) based insecticide resistance: The levels of oxidase activity in individual pests are relatively low and no reliable microtitre plate or dot-blot assay has been developed to measure p450 activity in single insects. The p450s are also a complex family of enzymes, and it appears that different cytochromes p450s produce resistance to different insecticides.

Molecular- Based methods for detection Insecticide resistance ……..
For DNA

PCR based

- RAPD
- AFLP
- MICRO SATELLITES
- SNP

NON – PCR based

- RFLP – SOUTHERN BLOT

For RNA

- RT –PCR
- DD –RT –PCR
- NORTHERN BLOT
- MICRO ARRAY

For Protein

- ENZYME ASSAY
- ELISA
- WESTERN BLOT
- SDS – PAGE
- MALDI – TOF

Brief gist of PCR: The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. There are three major steps involved in the PCR technique: denaturation, annealing, and extension. PCR is useful in the investigation and diagnosis of a growing number of diseases. Qualitative PCR can be used to detect not only human genes but also genes of bacteria and viruses. PCR is also used in forensics laboratories and is especially useful because only a tiny amount of original DNA is required. PCR can identify genes that have been implicated in the development of cancer. Molecular cloning has benefited from the emergence of PCR as a technique.

Blotting techniques: Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support generally nylon or nitrocellulose membranes.

Blotting of nucleic acid is the central technique for hybridization studies. Nucleic acid labeling and hybridization on membranes have formed the basis for a range of experimental techniques involving understanding of gene expression, organization, etc.

General principle: The blotting methods are fairly simple and usually consist of four separate steps: electrophoretic separation of protein or of nucleic acid fragments in the sample; transfer to and immobilization on paper support; binding of analytical probe to target molecule on paper; and visualization of bound probe. Molecules in a sample are first separated by electrophoresis and then transferred on to an easily handled support medium or membrane. This immobilizes the protein or DNA fragments, provides a faithful replica of the original separation, and facilitates subsequent biochemical analysis. After being transferred to the support medium the immobilized protein or nucleic acid fragment is localized by the use of probes, such as antibodies or DNA, that specifically bind to the molecule of interest. Finally, the position of the probe that is bound to the immobilized target molecule is visualized usually by autoradiography. Three main blotting techniques have been developed and are commonly called Southern, northern and western blotting

Colorimetric detection: The colorimetric detection method depends on incubation of the western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

Chemiluminescent detection: Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras which captures a digital image of the western blot. The image is analyzed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used.

Radioactive detection: Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest (see image to the right). The importance of radioactive detections methods is declining, because it is very expensive, health and safety risks are high and ECL provides a useful alternative.

Fluorescent detection: The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photo sensor such as CCD camera

equipped with appropriate emission filters which captures a digital image of the western blot and allows further data analysis such as molecular weight analysis and a quantitative western blot analysis. Fluorescence is considered to be among the most sensitive detection methods for blotting analysis.

Reverse Transcription PCR (RT-PCR): Reverse transcription polymerase chain reaction (RT-PCR) is one of many variants of polymerase chain reaction (PCR). This technique is commonly used in molecular biology to detect RNA expression. RT-PCR is often confused with real-time polymerase chain reaction (qPCR) by students and scientists alike. However, they are separate and distinct techniques. While RT-PCR is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA, qPCR is used to quantitatively measure the amplification of DNA using fluorescent probes. qPCR is also referred to as quantitative PCR, quantitative real-time PCR, and real-time quantitative PCR.

DD-RT-PCR (Differential display): Differential display (also referred to as DDRT-PCR or DD-PCR) is the technique where a researcher can compare and identify changes in gene expression at the mRNA level between any pair of eukaryotic cell samples. The assay may be extended to more than one pair, if needed. The paired samples will have morphological, genetic or other experimental differences for which the researcher wishes to study the gene expression patterns, hoping to elucidate the root cause of the particular difference or specific genes that are affected by the experiment

DNA-MICRO ARRAY: A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles (10−12 moles) of a specific DNA sequence, known as probes (or reporters or oligos). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Enzyme Linked Immunosorbent Assay (ELISA): The enzyme-linked immunosorbent assay is a test that uses antibodies and color change to identify a substance. ELISA is a popular format of "wet-lab" type analytic biochemistry assay that uses a solid-phase enzyme immunoassay (EIA) to detect the presence of a substance, usually an antigen, in a liquid sample or wet sample.

Conclusions

Biochemical techniques offer the capability of detecting the initial stages of resistance in a population and the mechanism of resistance involved. More sensitive tests combined with rapid confirmatory susceptibility tests will enhance the feasibility

of managing resistance through operational strategies. Pioneering experiments in the field should continue and should be promoted in both public health and agricultural situations.

Genetic definition of strains of key pests should be increased so that techniques can be developed based on biochemical mechanisms that are known with certainty. Presently linkage relationships for genes relevant to resistance are known only for strains of *Aedes aegypti*, *Drosophila melonogaster* and *Musca domestica*. Major agricultural pests must be studied genetically so that the powerful tools of molecular genetics can be applied to such problems as the regulation of resistance genes.

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Crop	pest	Resistance to
Brassicas	Green peach aphid *Myzus persicae*	Carbamates, Organophosphates, Pyrethroids-Pyrethrins, Neonicotinoids
	Diamondback Moth *Plutella xylostella*	Carbamates, Organophosphates, Phenylpyrazoles (Fiproles), Pyrethroids Neonicotinoids, Spinosyns, Avermectins-Milbemycins, Chlorfenapyr, Benzoylureas, Diacylhydrazines, METI acaricides, Indoxacarb, Metaflumizone, Diamides,
	Beet armyworm *Spodoptera exigua*	Carbamates, Organophosphates Phenylpyrazoles (Fiproles), Pyrethroids Neonicotinoids, Spinosyns, Avermectins-Milbemycins, Chlorfenapyr, Benzoylureas, Diacylhydrazines, METI acaricides and insecticides, Indoxacarb, Metaflumizone,
Cereals	English Grain Aphid *Sitobion avenae*	Pyrethroids-Pyrethrins
Corn and Cotton	Stink Bug *Euschistus Heros*	Carbamates, Organophosphates
Oilseed and Rape	Green Peach Aphid *Myzus persicae*	Carbamates, Organophosphates, Pyrethroids, Neonicotinoids
	Pollen Beetle *Meligethes aeneus*	Pyrethroids-Pyrethrins
Glasshouse vegetables	Melon & Cotton Aphid *Aphis gossypii*	Carbamates, Organophosphates, Pyrethroids, Neonicotinoids,
	Tobacco Whitefly *Bemisia tabaci*	Organophosphates, Cyclodiene organochlorines, Pyrethroids-Pyrethrins, Neonicotinoids, Pyriproxyfen, Pymetrozine, Buprofezin
	Western Flower Thrips *Frankliniella occidentalis*	Carbamates, Organophosphates, Cyclodiene organochlorines, Phenylpyrazoles (Fiproles), Pyrethroids-Pyrethrins, Neonicotinoids, Spinosyns, Avermectins-Milbemycins
	Green Peach Aphid *Myzus persicae*	Carbamates, Organophosphates, Pyrethroids-Pyrethrins, Neonicotinoids
	Beet Armyworm *Spodoptera exigua*	Carbamates, Organophosphates, Phenylpyrazoles (Fiproles), Pyrethroids-Pyrethrins, Neonicotinoids, Spinosyns, Avermectins-Milbemycins, Chlorfenapyr-DNOC-Sulfuramid, Benzoylureas, Diacylhydrazines, METI acaricides and insecticides, Indoxacarb, Metaflumizone
	Two spotted Spider Mite *Tetranychus urticae*	Carbamates, Avermectins-Milbemycins, Clofentezine-Hexythiazox-Diflovidazin, Organotin miticides, Acequinocyl, METI acaricides and insecticides,
	Tomato Leafminer *Tuta absoluta*	Pyrethroids-Pyrethrins, Avermectins-Milbemycins, Nereistoxin analogues, Benzoylureas, Indoxacarb
Soybean	Stink Bug *Euschistus Heros*	Carbamates, Organophosphates

Pome fruits	European Red Mite *Panonychus ulmi*	Organophosphates, Pyrethroids-Pyrethrins, Clofentezine-Hexythiazox-Diflovidazin, Organotin miticides, METI acaricides and insecticides
	Two spotted Spider Mite *Tetranychus urticae*	Carbamates, Avermectins-Milbemycins, Clofentezine-Hexythiazox-Diflovidazin, Organotin miticides, Acequinocyl, METI acaricides and insecticides
Potatoes	Green Peach Aphid *Myzus persicae*	Carbamates, Organophosphates, Pyrethroids-Pyrethrins, Neonicotinoids
Rice	Brown Plant hopper *Nilaparvata lugens*	Carbamates, Organophosphates, Cyclodiene organochlorines, Phenylpyrazoles (Fiproles), Pyrethroids-Pyrethrins, Neonicotinoids, Buprofezin
Solanaceous crops	Colorado Potato Beetle (*Leptinotarsa decemlineata)*	Carbamates, Organophosphates, Pyrethroids-Pyrethrins, Neonicotinoids
Humans and animals	Housefly *Musca domestica*	Carbamates, Organophosphates, Cyclodiene organochlorines, Pyrethroids-Pyrethrins, DDT-Methoxychlor, Neonicotinoids, Spinosyns, Avermectins Milbemycins, Pyriproxyfen, Benzoylureas, Cyromazine, Indoxacarb,
	Sandfly *Sandfly species*	Carbamates, Organophosphates, DDT-Methoxychlor

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Comparison of Southern, Northern, Western blot techniques

Particulars	**Southern blot**	**Northern blot**	**Western blot**
Invented by	Edwin Southern	Alwine *et al.*	Towbin & Co workers
Year of invention	1975	1977	1979
Other term used	Southern hybridization, southern blotting	Northern blot tech. & Northern hybridization	Western blotting, Immunoblotting
Used for	Searching for **gene** of interest	Searching an **mRNA** – of interest	Searching for **protein** of interest
Probe used	Labelled DNA probe is used	Labelled DNA probe is used	Antibody is used as probe
Restriction enzyme	Used to cut the DNA	Not used	Not used
Denatured used	NAOH containing alkaline soluti0on is used	Formaldehyde is used	Not used
Antibody	Not used	Not used	Detect the protein of interest
+ signal indicates	DNA segment is containing gene of interest	RNA of interest is present	Protein of interest is present
Information is obtained about	Presence / absence of gene of interest	Expression of gene of interest	Presence of protein of interest
Digestion	DNA is digested before use	-------	Protein is not digested before use

Comparison of Different Molecular Techniques

Features	RFLPs	RAPDs	SSRs	AFLPs	SNPs
Technical basis	Sequence specific endonuclease restriction, southern blot and hybridization	DNA amplification with random decamer primers	DNA amplification of simple sequence re-peats of different length using specific primers	Endonuclease restriction use of adopters and selective primers	Sequence comparison and analysis
Type of polymorphism	Single base changes	Single base changes	Changes in repeat length	Single base changes	Single base changes
Loci analyzed per primer/attempt	1–4	1–50	1–4	20–100 or more	1
Detection	Radio labeled DNA probe	Ethidium bromide staining	Ethidium bromide or silver staining	With or without radio labeled DNA probe	Sequencing DNA fragments
Inheritance	Co-dominant	Dominant	Co-dominant	Dominant	Dominant
Ease of use	Cumbersome	Easy	Easy	Not cumbersome	Easy
Need for sequence information	No	No	Yes	No	Yes
Reproducibility	High	Unreliable	High	High	High
Development cost	Very high	low	high	high	high
Cost per analysis	high	low	low	moderate	Low
Amenable to automation	low	moderate	high	moderate	High
PCR based	no	yes	yes	yes	yes
DNA-required	10 µg	0.02 µg	0.05 µg	0.5–1.00 µg	0.05 µg
DNA quality required	High	low	moderate	High	high