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Review

Immunodiagnosis of pesticides: A review

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The word 'pesticide' is known worldwide for repelling or killing all kinds of pests in both agricultural and domestic settings but the harmful effects they cause on the immediate environment and living beings exposed to them has raised serious concerns and makes it more necessary to detect the minutest levels of contamination. Convenient, cost-effective and rapid pesticide detection systems are urgently needed, for which immunoassays are the best option. The present review deals with the immunodiagnosis of pesticides; various steps involved in development of an immunoassay focussing primarily on hapten preparation and conjugation strategies, generation of antibodies and assay optimization techniques in enzyme linked immunosorbent assay (ELISA) format. Their advantages over conventional methods and limitations have been discussed, followed by a bird's eye view of the multianalyte detection, immunosensors and other options-to-be-explored for better and more sensitive detection of pesticides.

Key words: Immunodiagnosis, pesticides, immunoassay.

INTRODUCTION

Pesticides are widely used for killing, preventing, destroying, repelling or mitigating insect pests, weeds, rodents, fungi, and other organisms that compete for food supply or threaten public health, cause nuisance and affect national economies, thereby giving a plenty of benefits to man and environment. More than 20000 pesticide products with nearly 900 active ingredients are registered by United States Environment Protection Agency (USEPA) for use as insecticides, miticides, herbi-cides, rodenticides, nematicides, fungicides, fumi-gants, wood preservatives and plant growth regulators (WHO, 2004). Their use helps in improving mankind by preventing food loss, making it more available to man, with longer storage life and lower costs. Though pesti-cides provide a relief to the mankind from pests, they have certain significant economic, environmental and public health impacts also. Widespread use of pesticides has raised sincere concerns over food and environmental contamination caused by them. A leading survey done by USEPA (Grube et al., 2011) revealed an approximate 5.6 billion pounds of annual pesticide usage worldwide. WHO (2009) estimated that globally, every year, 3 million people suffer health effects from exposure to pesticides and a minimum of 300,000 people die, of which, 99% belonging to low- and middle- income countries.

Several groups of pesticides are used commercially namely: Organophosphates, organochlorines, carbamates and pyrethroids. Organochlorines possess a chlorinated hydrocarbonmoeity;dichorodiphenyltrichloroethane(DDT), dieldrin, endosulfan being the common ones. Organo-

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Abbreviations: DDT, Dichorodiphenyltrichloroethane; **OP's,** organophosphate pesticides; **LC,** liquid-chromatography; **HPLC,** high performance liquid chromatography; **GPC,** gel permeation chromatography; **GC-MS,** gas chromatography-mass spectrometry; **LODs,** limits of detection; **TCP,** 3,5,6-trichloro-2-pyridinol; **ELISA,** enzyme linked immunosorbent assay; **TLC,** thin layer chromatography; **NMR,** nuclear magnetic resonance; **BSA,** bovine serum albumin; **OVA,** ovalbumin; **CAMM,** computer-assisted molecular modelling; **scFv,** single-chain variable fragment.

phosphate pesticides (OP's) are characterized by the presence of a phosphate-ester moiety in their chemical structure and account for approximately half of the pesticides used worldwide (ENVIS-NIOH, 2007). Their ability to degrade easily makes them an attractive alternative to the organochlorine pesticides, such as DDT, aldrin and dieldrin (Needleman, 2005) but OP's have greater acute toxicity, posing risks to people who may be exposed to large amounts (Costa, 2006). Commonly used organophosphates include parathion, malathion, chlorpyrifos. Pyrethroids are synthetic derivatives of pyrethrin and include cypermethrin, deltamethrin, among others. Another category includes carbamate pesticides, the derivatives of carbamic acid with carbaryl, bendiocarb, methomyl and propoxer dominating the category. Organophosphates and carbamates primarily act as neurotoxincants; they inhibit the enzyme acetylcholine-esterase, resulting in oversignalling, which leads to muscle paralysis and even death.

The dose of a pesticide is important in predicting the potential toxicity. Further, factors such as age, health and possibly gender may significantly lower the threshold for toxic effects. Even a single exposure to pesticide poses risks to man and other mammals, birds, fish and aquatic invertebrate species. Prolonged and multiple exposures to toxic concentrations raises the risks to wildlife as has been reviewed extensively by Parsons et al. (2005). Because of the widespread use of pesticides in agricultural and residential areas and the considerable toxic ill effects these cause on the persons exposed to it even at low levels, their detection even at minutest levels becomes a major concern. Hence the present review has been made with an aim to compile the various techniques used for detection of pesticides and concisely evaluate the recently developed state-of-the-art immunodiagnostic techniques over the conventional ones.

CLASSICAL TESTS USED TO DETECT PESTICIDES

Conventionally, gas-chromatography (GC), liquidchromatography (LC), high performance liquid chromatography (HPLC) and gel permeation chromatography (GPC) have been used with great ease and success for detection of pesticides. Zhu et al. (2006) applied gas chromatography-mass spectrometry (GC-MS) with negative chemical ionization (NCI) for the determination of five pyrethroid pesticide residues (fenpropathrin, cyfluthrin, fenvalerate, deltamethrin, cypermethrin) in traditional Chinese medicinal plants and obtained limits of detection (LODs) from 0.5 to 5 ppb with recoveries ranging from 70 to 120%. The method was considered rapid, sensitive and suitable for the analysis of large numbers of samples simultaneously. A deadspace solid-phase microextraction method developed by Chai et al. (2008) for the determination of various pesticides in vegetables and fruits by gas chromatography with an electron capture detector resulted in more than 80% recovery for all the investigated samples. In another study, ultrasonic-assisted dispersive liquid-liquid microextraction (UA-DLLME) followed with GC- flame ionization detection (FID) was applied for the analysis of cypermethrin and permethrin residues in pear juice (Du et al., 2010). The enrichment factors for cypermethrin and permethrin residues were 344 and 351, respectively and LODs ranged from 3.1 to 2.2 µg/kg, recoveries were 92.1 to 107.1%. The concentration of acephate, methamidophos and omethoate was determined in animal and fishery products and their processed foods and honey (Jia et al., 2010) using extraction of target analytes with ethyl acetate and pre-purification on a primary secondary amine (PSA) mini-column followed by detection using LC-MS in the electrospray ionizaton mode with recoveries of the method ranging between 71.4 and 98.4% ± 12.5%. Organochlorine pesticides residues have been determined in commercial fruit species by Cieslik et al. (2011) where target compounds were extracted and cleaned by QuEChERS (quick, easy, cheap, effective, rugged and safe) method followed by GC-MS analysis. The recovery rates of the method ranged from 70 to 120 ±17% in the majority of cases; limit of quantification varied between 0.001-0.013 mg/kg. An accelerated solvent extraction system (HPLC with GPC) was used for separation, detection, and quantification of the pesticides from the sediment-sample extracts followed by their detection by GC/MS (Hladik and McWayne, 2012). Recoveries in test sediment samples fortified at 10 µg/kg dry weight ranged from 75 to 102%; relative standard deviations ranged from 3 to 13%, LODs ranged from 0.6 to 3.4 µg/kg dry weight.

However, determination of metabolites cannot be taken as standard for estimating the exposure to a pesticide, since residues of metabolites frequently occur on fruits and vegetables, often at concentrations 10-20 times greater than the parent pesticide. Besides, structurally related pesticides can also be converted to same metabolites. A bacterium strain KR100 isolated from a Korean rice paddy soil by Kim and Ahn (2009) is able to degrade chlorpyrifos as well as chlorpyrifos-methyl to 3,5,6-trichloro-2-pyridinol (TCP). Moreover, conventional methods owe their high cost for set-up skilled labour and extensive sample preparation steps, so it becomes necessary to replaced them with more analytical and simpler techniques for which immunoassays are a good option. In an attempt to compare the efficacy of immunoassays with classical tests, Curwin et al. (2010) collected urine samples from 51 participants among farm families in Iowa to analyze the status of exposure to chlorpyrifos using enzyme linked immunosorbent assay (ELISA) and HPLC in tandem mass spectrometry. The immunoassay methods consistently had significantly higher geometric mean estimates for the metabolites. The LOD for TCP as a metabolite of chlorpyrifos ranged from 13.9-14.13 µg/L by HPLC and 2.91-2.99 µg/L from

immunoassay. The study confirmed that HPLC-MS/MS methods tend to be less sensitive, costly and time consuming as compared to immunoassays and also require sample pre-treatment. The same has been proved by Rubio et al. (2003) who compared ELISA and HPLC for the detection and quantification of glyphosate-spiked Nanopure, tap, and river waters. The ELISA had a detection limit 0.6 ng/mL whereas the HPLC method had a detection limit of 50 ng/mL.

RECENTLY DEVELOPED IMMUNOASSAYS AND THEIR ADVANTAGES OVER CLASSICAL TESTS

The development of immunochemical techniques revolutionized the practice of pesticide analysis in veterinary medicine, agriculture, clinical chemistry and other areas including environmental and food analysis and have begun to gain acceptance as rapid, simple, sensitive, specific and cost-effective tools. These can detect nano-gram scale *in situ*. Immunoassays rely on detection of analyte concentration in the sample by measurement of level of antigen-antibody binding. Highly sensitive detection of analytes can be made by enzyme immunoassays such as ELISA. Development of an immunoassay typically follows some basic strategic principles in which antigen-specific antibodies are generated and then used to capture the antigen present in a biological sample or vice versa. Availability of antibodies with the desired affinity and specificity is a prerequisite for the development of immunoassays and the foremost step in antibody production is the preparation of an immunogen with molecular weight appropriate enough to target the animal's immune response. The major steps in the development of an immunoassay include: Preparation of hapten and immunogen and its characterization; conjugation of hapten to macromolecular carrier and its purification; immunization of host animals for generation of antibodies and optimization of the assay.

Preparation of hapten and immunogen

Most pesticides are unable to elicit an appropriate immune response because of their low molecular weight. Hence, production of antibodies against pesticide-hapten can be made possible by covalent linking of the hapten to a carrier protein, through a coupling spacer, to synthesize the 'immunogen'. Hapten immunochemistry occupies a major role in the area of development of antibodies. The major points of influence in the preparation of an immunogen include site of coupling to the carrier, the length of coupling spacers, the selection of optimized carriers, the coupling procedure as well as the number of haptens bound to one carrier molecule (Dankwardt, 2001). It is optimal to use C_3-C_6 spacers; too long spacers may bend back over the carrier and prevent proper exposure of the hapten. A wide variety of immunogens and protein/enzyme conjugates have been prepared and used in immunoassays for various pesticides. Basic synthetic ways of preparation of the hapten derivatives (hapten design) have been explored (Figure 2) by Franek and Hruska (2005) and Tong et al. (2007). Out of various ways of preparing a conjugate, it is important to select the best, which can elicit maximum immune response.

Structural modification of pesticide

Generally the haptens are desired to contain (a) a reactive group (b) an aromatic ring (c) branch atom or (d) heteroatom ring. The hapten needs a reactive functional group such as -COOH, -NH_2 or -OH to directly couple with the carrier proteins. Else, it has to be modified first to introduce at least one of these reactive groups in its structure to couple with the protein. For example, as described by Manclus et al. (1994, 1996) there are two approaches to prepare chlorpyrifos-hapten depending on the site of attachment of spacer arm: modification of the aromatic ring (Hapten 1) viz. spacer coupling through the pyridyl ring by substitution of chlorine at the 6th position (ortho- to the nitrogen) or the thiophosphate group (Hapten 2) through replacement of the o-ethyl group with a suitable spacer arm keeping the pyridyl ring moiety intact (Figure 1).

Characterization of the hapten

The hapten can be characterized by thin layer chromatography (TLC) or nuclear magnetic resonance (NMR) spectral analysis. TLC is a low-cost, maintenance-free, fast, and reliable method and also uses limited volumes of solvents; NMR offers better solutions in terms of confirmation.

Coupling of hapten to macromolecular carriers

Various approaches are used to couple the hapten to the carrier depending upon the chemical structure of haptens (Figure 2) namely: (1) Carboxyl-containing haptens can be coupled with the carrier using N-hydroxysuccinimide active ester/carbon-diimine or Woodward reagent protocol; (2) Amino-containing haptens can be linked to carriers by employing glutaraldehyde, diisocyanate, halo-nitrobenzene, hiophosgenation, diimine ester, or diazotization protocol; (3) Hydroxyl-containing haptens can be directly connected to the carrier through succinic anhydride or azobenzoic acid protocol; (4) Carbonyl-containing haptens (ketone or aldehyde) are usually connectd to carriers using amino-ox-acetic acid protocol; (5) Homogeneous or heterogeneous difunction reagents

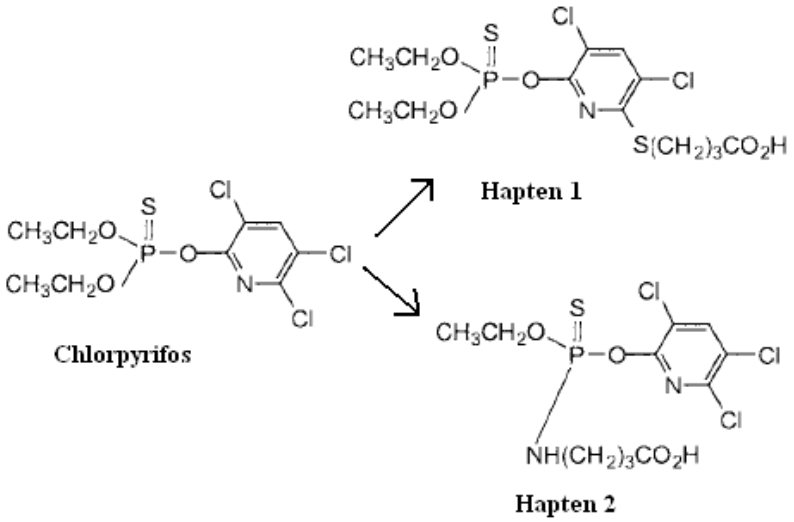

Figure 1. Synthesis of chlorpyrifos hapten (introduction of carboxylic group).

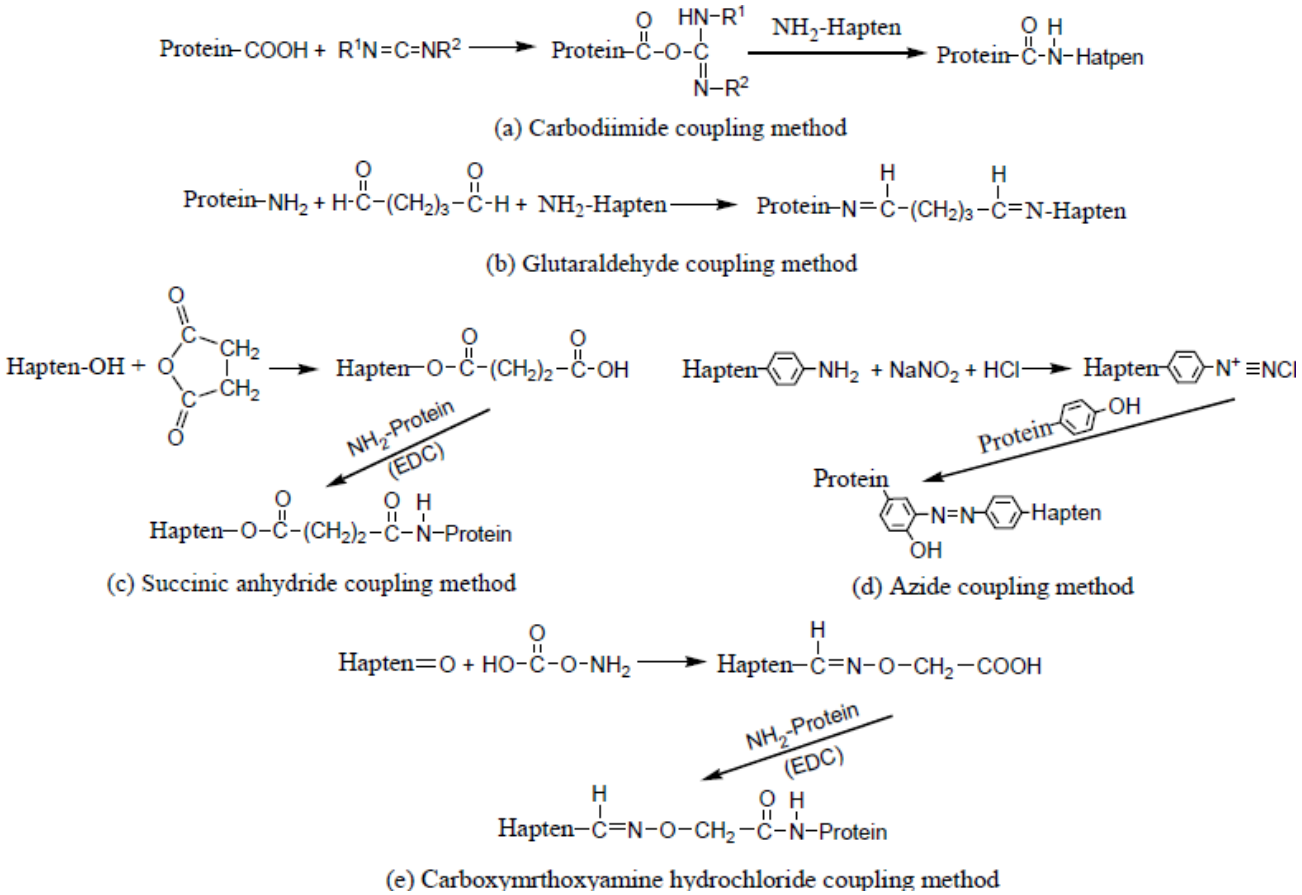

Figure 2. General principles of coupling of hapten to carrier protein (Tong et al. 2007).

can be used to synthesize the immunogen for mercapto-containing haptens (Tong et al., 2007).

Various proteins have been used by a number of analysts with ease and effectiveness, as carrier molecules with a variety of pesticides viz. bovine serum albumin (BSA) (Cho et al., 2002), keyhole limpet hemocyanin (KLH)

(Lee et al., 2003) and ovalbumin (OVA) (Zhang et al., 2008, Qian et al., 2009). A single polypeptide chain of BSA has 59 lysine groups containing side chain amino groups, out of which 30-35 are available for coupling to carboxyl group of hapten, which makes it an excellent carrier; OVA has 20 lysines and KLH has a very high number of lysines (300-600 are usually available for binding). It is always best to choose the carrier protein containing the optimum number of lysine residues for binding high number of binding sites in tertiary structure of the protein by masking the essential free amino groups. Moreover, the easy availability of BSA and its ability to solubilize in organic solvents under various pH range and ionic strength makes BSA a popular carrier protein. Also non-proteinaceous carriers viz. liposomes and dextran or synthetic molecules designed with appropriate functional groups such as poly-L-lysine and polyethylene glycol can be used as carriers in pesticide immunoassays.

However, the above trial and error-based procedures of hapten designing may give rise to antibodies lacking some features necessary to develop a useful immunoassay, besides being time-consuming and arduous. Immunochemists have recently come up with the computer-assisted molecular modelling (CAMM) as a useful tool for hapten design. CAMM offers assistance in predicting the spatial and electronic effects of molecular structure of hapten on its biological activity that are difficult or otherwise impossible to obtain, and can then be successfully applied in improving the sensitivity of immunoassays. Molecular modelling studies have been used to identify the best out of various potentially immunizing haptens for development of sensitive immunoassays against trichlorophenol (Galve et al., 2002), permethrin (Ahn et al., 2004), parathion (Liu et al., 2007) and semicarbazide (Vass et al., 2008). Applications of CAMM in hapten designing for immunoassay development along with limitations and prospects have been reviewed in detail by Xu et al. (2009). Xu et al. (2010) applied CAMM to model the hapten design to develop a broad-specific competitive indirect ELISA for fourteen O,O-diethyl organophosphorus pesticides. CAMM is foreseen as a practical and potential tool in rapid and more economical development of immunoassays.

Purification of conjugate

The conjugate to be used as immunogen needs to be obtained in the pure form to avoid production of nonspecific antibodies and unwanted cross-reactivities of the polyclonal antisera. The conjugate is separated from the uncoupled haptens by dialysis or gel filtration. Dialysis results in a well purified antigen and is a simple process and has been successfully used for separation of hapten-protein conjugates from uncoupled haptens for Acephate (Lee et al., 2003) and Fenthion (Zhang et al., 2008); bromophos hapten-protein conjugates were separated from the uncoupled haptens by gel filtration (Sephadex G-25), the same has been applied for the separation of chlorpyrifos-protein conjugates (Manclus et al., 1996). Reports are also available describing the separation of cyanophos-protein conjugates by gel filtration followed by dialysis (Park et al., 2002b).

PRODUCTION OF ANTIBODIES

Various warm blooded animals viz. mice, rabbit, goat or chicken can be immunized with the hapten-protein conjugate for pesticide. Rabbit is most widely used as they are easy to handle, respond quickly, produce adequate amount of antiserum and have a sufficiently long life span. Both polyclonal and monoclonal antibodies have been used for development of pesticide immunoassays so far. Though mAbs produced from hybridoma culture (Manclus et al., 1994, 1996) in laboratories offer the advantages of a steady supply and unvarying characteristics, many pesticide immunoassays still employ polyclonal ones (Cho et al., 2002; Brun et al., 2005) owing to the great effort and expense involved in mAb production. There is no standard protocol for immunization; generally, the animal is injected with small volumes of the inoculum at multiple sites and the immunization is repeated with booster doses at regular intervals (week or so). The animal is bled after each booster dose and antisera is collected.

A third possibility of obtaining antibodies is using recombinant antibody engineering techniques- that represent the next generation immunoreagents. Here, *in vitro* production of recombinant antibodies (rAbs)- or their fragments (for example, scFv or Fab) (Figure 3) is made by creating libraries of antibody gene segments followed by phage display, ribosomal display or yeast display, from which antibodies of desired specificities and affinities tailored by site-directed mutations can be selected. Single-chain variable fragment (scFv) antibodies have been produced by phage display against a number of pesticides including parathion (Chambers et al., 1999) methamidophos (Li et al., 2006) and fenitrothion (Luo and Xia, 2012).

DEVELOPMENT OF ASSAY

The antibodies so obtained can be used to develop immunoassays in different choices of formats. These may be broadly categorized as "competitive" and "non-competitive", depending upon their utilization of limited concentration of available antibody while non-competitive assay rely on the measurement of antibody binding sites getting occupied after they are allowed to react with the analyte; competitive assay is based on the competition between solid-phase (bound) and soluble antigen for

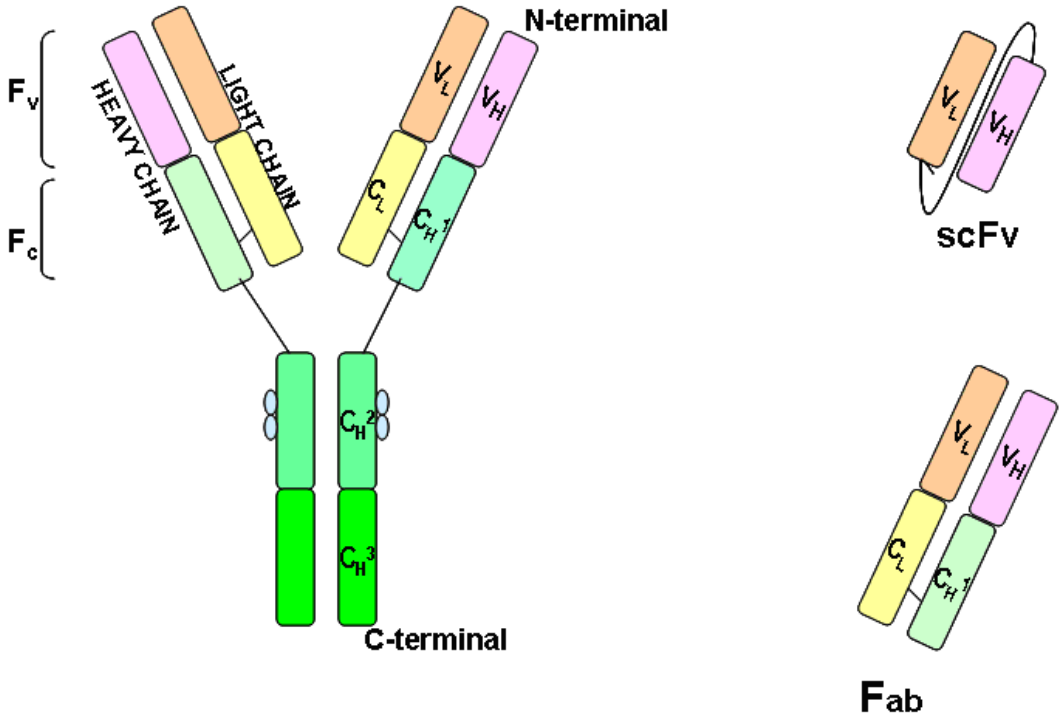

Figure 3. Structure of a typical antibody and antibody fragment types. C_H, Constant heavy weight; C_L, constant light weight; V_H, variable heavy chain; V_L, variable light chain; Fab, antigen binding fragment; scFv, single chain variable fragment.

limited antibodies and give an indication of the antibody binding sites left unoccupied and is more preferred for pesticide detection. With increase in concentration of pesticide added for competition, lesser amount of antibodies is available for binding with the bound antigen and hence there is a decrease in absorbance. A highly sensitive immunoassay will have a low detection limit (DL) and that calls for high affinity between the antibody and the analyte. Dankwardt (2001) presents a complete overview of the immunoassays developed for detection of various pesticides. Table 1 summarises the work done after 2001 by various immunochemists in development of highly sensitive ELISAs against various pesticides along with their limit of detection (LOD) and I_{50} values.

Enzyme assays have been used for the rapid screening of insecticides, but these assays may lack distinction between similar pesticides. Many workers have found cross-reactivity of ELISA developed for one pesticide towards certain other pesticides of the same category, probably due to structural similarity or same functional groups that act as antibody competitors or even against the carrier protein used for hapten-conjugation. So it is always essential to check the immunoassay for potential cross reactivities before applying. This is usually done by comparing the standard curves of the analyte under investigation with similar haptens, using analyte concentrations at 50% of the inhibition curve as the reference. Cho et al. (2002) found the ELISA for chlorpyrifos showed 66.6% cross reactivity with chlorpyrifos-methyl, 15.6% with bromophos-ethyl, 4.58% with bromophos-methyl and 3.05% with dichlofenthion. Park et al. (2002c) found the ELISA developed for bromophos to show cross-reactivity with chlorpyrifos and fenitrothion because of their similar aromatic structure. Brun et al. (2005) found the ELISA developed against chlorpyrifos to be cross-reactive against chlorpyrifos-methyl, bromophos-methyl and fenchlorphos. The commercial magnetic particle-based ELISA kit used by Sullivan et al. (2007) for the detection of chlorpyrifos showed cross-reactivity with chlorpyrifos-methyl (37%), while reactivity with other pesticides ranged from 1.6 to 10.7%.

CONCLUSIONS AND FUTURE PROSPECTS

ELISA can be potentially adopted as a routine test for the detection of pesticides for rapid and cost-effective mass screening of a large number of samples simultaneously.
So far immunoassays dominate the field of pesticide diagnosis, being the highest sensitive tests. Dipstick immunoassays based on competitive immunoassay format have great potential to become cost-effective and sensitive tool for on-site monitoring of pesticides. Its main advantage is its ease and effectiveness to use for field analysis and high sensitivity. Quantification of the pesticides

Table 1. List of ELISAs developed for detection of various pesticides (2002 onwards).

Pesticide	Antibody used	LOD	I_{50}	Reference
Chlorpyrifos	pAb	0.1 ng/mL	20 ng/mL	Cho et al. (2002)
Bromophos-ethyl	pAb	1.0 ng/mL	6.5 ng/mL	Kim et al. (2002)
Deltamethrin	pAb	1.1 ± 0.5 µg/L	17.5 ± 3.6 µg/L	Lee et al. (2002)
2-(2,4,5 trichloro phenoxy) propionic acid	pAb	0.05 µg/L	0.80 µg/L	Morais et al. (2002)
Isofenphos	pAb	70 ng/mL	580 ng/mL	Park et al. (2002a)
Bromophos	pAb	7 ng/mL	40 ng/mL	Park et al. (2002c)
Fenitrothion	pAb	0.3 ng/mL	6 ng/mL	Watanabe et al. (2002)
Fenthion	pAb	0.1 µg/L	1.2 µg/L	Cho et al. (2003)
Acephate	pAb	2 ng/mL	25 ng/mL	Lee et al. (2003)
Fenthion	pAb	0.03 ng/ml	0.05 ng/ml	Brun et al. (2004)
Isoproturon	pAb mAb	0.1 µg/L 0.003 µg/L	1.06±0.34 µg/L 0.07±0.04 µg/L	Kramer et al. (2004)
Cypermethrin	pAb	1.3 ± 0.5 µg/L	13.5 ± 4.3 µg/L	Lee et al. (2004)
Chlorpyrifos	pAb	0.3 ng/mL	271 ng/mL	Brun et al. (2005)
Cyhalothrin	pAb	4.7 µg/L	37.2 µg/L	Gao et al. (2006)
Pirimiphos-methyl	mAb	0.07 ng/mL	4.2 ng/mL	Yang et al. (2006)
Imidacloprid	pAb	30 ng/mL	995.4 ng/mL	Li et al. (2007)
Triazophos	mAb	0.02 µg/L	0.21 µg/L	Liang et al. (2007)
Pentachloronitrobenzene	pAb	7 ng/mL	37 ng/mL	Xu et al. (2007)
Triazophos	mAb	0.36 to 7.89 ng/mL	1.69 ng/mL	Jin et al. (2009)
Carbofuran	mAb	1.89 to 45.95 ng/mL	9.32 ng/mL	
2,4-dinitroaniline	mAb	0.05±0.03 µg/L	0.24±0.06 µg/L	Kramer et al. (2008)
2,6-dinitroaniline	mAb	0.11±0.08 µg/L	0.61±0.08 µg/L	
O-ethyl o-4-nitrophenyl phenyl phosphonothioate	pAb	0.9 ng/mL	8.4 ng/mL	Shim et al. (2008)
fenazaquin	pAb	1.8 ng/mL	42.13 ng/mL	Kyung et al. (2009)
Parathion	pAb	0.15 ng/mL	0.95 ng/mL	Liu et al. (2009)
Deltamethrin	mAb	1.2±1.3 ng/mL	17.0±3.3 ng/mL	Kong et al. (2010)
EPN	mAb	0.09 ng/mL	0.6 ng/mL	Shim et al. (2010)
Atrazine	pAb	0.1 ng/mL	17.5 µg/mL	El-Gendy et al. (2011)
Chlorpyrifos	mAb	0.1 ng/mL	3.3 ng/mL	Liu et al. (2011a)
Pretilachlor	pAb	6.9 ng/L	0.0359 mg/L	Liu et al. (2011b)
Imidaclothiz	mAb	0.0178 ± 0.0018 mg/L	0.0875 ± 0.0034 mg/L	Fang et al. (2011)
Triazophos	mAb	0.063 ng/mL	0.87 ng/mL	Jin et al. (2012)
Imidacloprid	pAb	0.03-0.16 ng/mL	1.2-3.0 ng/mL	Wang et al. (2012)

is carried out by measuring the dot colour by spectronic read-out or naked eye (Gabaldon et al., 2003). However the errors inherent in immunoassays may give false positives and lack absolute specificity as was originally presumed, besides exhibiting cross reactivity with similar epitopes, thereby misleading the results. But an approximation of the results can definitely be inferred from immunoassays, which can further be verified.

Another limitation of immunoassays is their restriction to detect single analyte per assay. Hence, new approaches for development of multianalyte immunoassays are being undertaken, for the advantages of simultaneous detection of multiple analytes in a single assay, high sample throughput, reduced sample consumption and lower cost per assay they offer. Important progress is expected in the field of ‘ambient analyte immunoassays’ based on Microspot detection systems. These systems rely on "ratiometric" analysis, involving measurement of analyte concentration from the ratio of signals emitted by two labelled antibodies- "Sensor" antibody, deposited as a microspot on a solid support which is exposed to the analyte-containing sample; and "Developing” antibody directed against either occupied or unoccupied binding sites of the sensor antibody. An array of sensor antibodies of different selectivity can be employed in the form of a chip and the fluorescent signal ratio emitted

from each discrete antibody couplet in the array can be used for multianalyte determination.

Gold- or selenium-nanoparticle based aggregation immunoassay technique is also being recently undertaken for multianalyte sampling of the pesticides. An enzyme immunoassay is performed on the antibody-nanoshell conjugates and aggregates of analytes of various morphologies present in the complex sample medium are formed on their surface thereby allowing simultaneous read-out of multiple analytes. A gold nanoparticle (GNP) based dipstick competitive immunoassay has been developed for several pesticides viz. an LOD of 1ng/mL has been developed for atrazine (Kaur et al., 2007), 27 ng/mL for DDT (Lisa et al., 2009), 3 ng/mL for 2,4-dichlorophenoxyacetic acid (Boro et al., 2011); though Salmain et al. (2008) found no significant difference in the IC_{50} and LOD values obtained from gold particle based immunosensor and the competitive inhibition ELISA using the same antibody and the antigen.

In order to increase their range, speed and sensitivity, recently ELISA techniques have been combined with biosensors, to form immunosensors, in which analyte concentration is directly determined by measuring the alteration in physical properties (electrical or optical) induced by the formation of an immune complex between the analyte and the antibody immobilized on the transducer carrier surface acting as a sensing device. Till date, immunosensors based on optical, piezoelectric (PZ), electrochemical and micromechanical designs have come up as the most sophisticated immunoassay format to detect trace amounts of pesticides (Suri et al., 2009). Opto-electronic based biosensors such as surface plasmon resonance (SPR) sensors, interferometer devices or grating couplers offer the benefits of real-time measurement of biomolecular interactions, portability, versatility and regenerability in multi-analyte detection. An SPR-based biosensor has been recently developed for multianalyte detection of sulfonamides (Bienenmann-Ploum et al., 2005) and for simultaneous detection of DDT, chlorpyrifos and carbaryl (Mauriz et al., 2007). Overall, it would not be wrong to say that despite the advances made in developing sensitive diagnostic tools for detection of pesticides, the quest for an even better one still goes on; the requirement for novel signal detection equipments still persists; the need for better and more sophisticated assays for pesticide monitoring in environmental and food analysis is call of the hour.

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