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Immunoassay-based approaches for development of screening of chlorpyrifos

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Abstract

Chlorpyrifos (CPF) is an extensively used organophosphate pesticide for crop protection. However, there are concerns of it contaminating the environment and human health with estimated three lakh deaths annually. Detection of CPF in blood samples holds significance to avoid severe health outcomes due to continuous exposure. The most common techniques for CPF detection are Gas chromatography (GC) and high-performance liquid chromatography (HPLC). However, these techniques might not be feasible at the community healthcare level due to high-cost instrumentation, time-consuming sample preparation protocol and skilled analysts. Therefore, rapid, effective and economical methods such as immunoassay would be imperative for CPF detection in biological samples. The vital step in immunoassay development is the design of a potent immunogen from non-immunogenic molecules. The molecular modelling protocol could assist in redesigning known CPF linkers and inserting them at different substitutable positions of CPF to get distinctive CPF derivatives. Molecular docking and binding free energy analysis can be used to identify the CPF derivatives having a better binding affinity with carrier protein compared to CPF. The top-ranked CPF derivatives based on docking score and binding energy could be ideal for synthesis and immunogen development. The present review will comprehend technological trends in immunoassay kits for detecting chlorpyrifos from biological samples.

Keywords: Chlorpyrifos, Gas chromatography, Immunoassay kit, Molecular modelling, Organophosphorus, Pesticides

Introduction

Pesticides are chemical compounds, such as insecticides, fungicides, molluscicides, rodenticides and nematicides, used to kill or control the organisms, fungi and other such life forms that not only damage the irrigated crops and vegetables but cause ill effects to domestic animals and human beings (Amaral AFS 2014; Bhadekar et al. 2011; Dar et al. 2019) (Fig. 1). Pesticides improve agrarian production, food quality, and the financial status of cultivars. However, the unrestrained application of pesticides has caused environmental distress/degradation propagating to several health concerns (Ragnarsdottir 2000).

Organophosphorus (OPs) compounds are among one of the commonly used pesticides for farming and domestic purposes. OPs have moderately low persistence in the atmosphere but their usefulness is counterbalanced by severe toxicity to human well-being and the ecosystem (Mauriz et al. 2006). CPF is considered to be an insecticide which has quite a broad spectrum and it has been used quite frequently to prevent the growth of different insect species and specifically the arthropods on crops which are important to mankind. In urban settings, it is viably utilized on lawns, ornamental plants and pet items. CPF is a fluid material in its regular state

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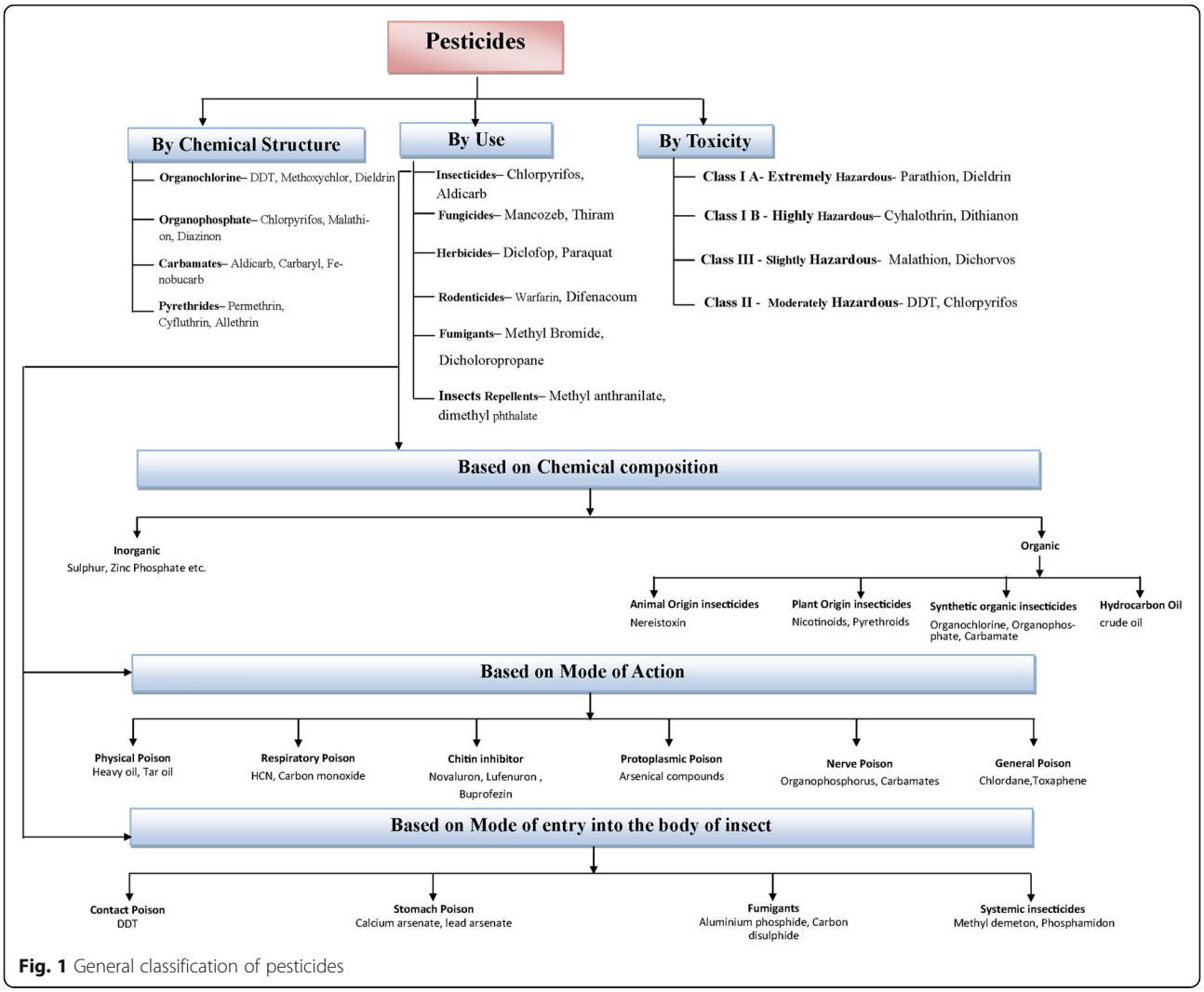


Fig. 1 General classification of pesticides

and latent exposure occurs through inhalation, ingestion and dermal absorption (Fig. 2). Subsequently, its exposure could lead to acute to chronic health issues such as paresthesia, light-headedness, tachycardia (Rathod and Garg 2017), cancer (Alavanja and Bonner 2012), neurological diseases (Grandjean and Landrigan 2014), adverse reproductive outcomes (Rim 2017) and respiratory diseases (Nicolopoulou-Stamati et al. 2016). Thus, monitoring of OP residues can be considered a significant step for the welfare of human society and it should be regulatory control at the national and international trading levels.

With recent advances in research, many techniques are available which helps in the qualitative analysis of pesticides; few of the very helpful techniques are gas and high-performance liquid chromatography (HPLC), LC-MS (Ravelo-Pérez et al. 2006; Stachniuk and Fornal 2016). These techniques are commonly used for monitoring pesticides but have so many drawbacks such as high-cost instruments, time-consuming procedures, require skilled analysts and at the same time not well versed with the on-spot analysis of different parameters. In contrast, when it comes to high number samples and in few cases where on-site screening is required, immunoassays are one of the most powerful techniques (Beyene et al. 2019). In this regard, immune assay-based kits could be useful for mass screening of CPF concentration at the community level.

Immunoassays are a quantitative technique which utilizes the specific interaction between specific epitopes present on antigens and the binding affinity of antibodies; they help in the detection and measurement of large-size molecules like proteins as well as small molecules (drugs, pesticides) (Wang et al. 2018). Immunoassay, viz. ELISA (enzyme-linked immunosorbent assays), has simplicity, cost-effectiveness, high selectivity and specificity in detecting analytes from biological or environmental specimens, hence are being widely used for pesticide detection in recent times (Ivanov 2019).

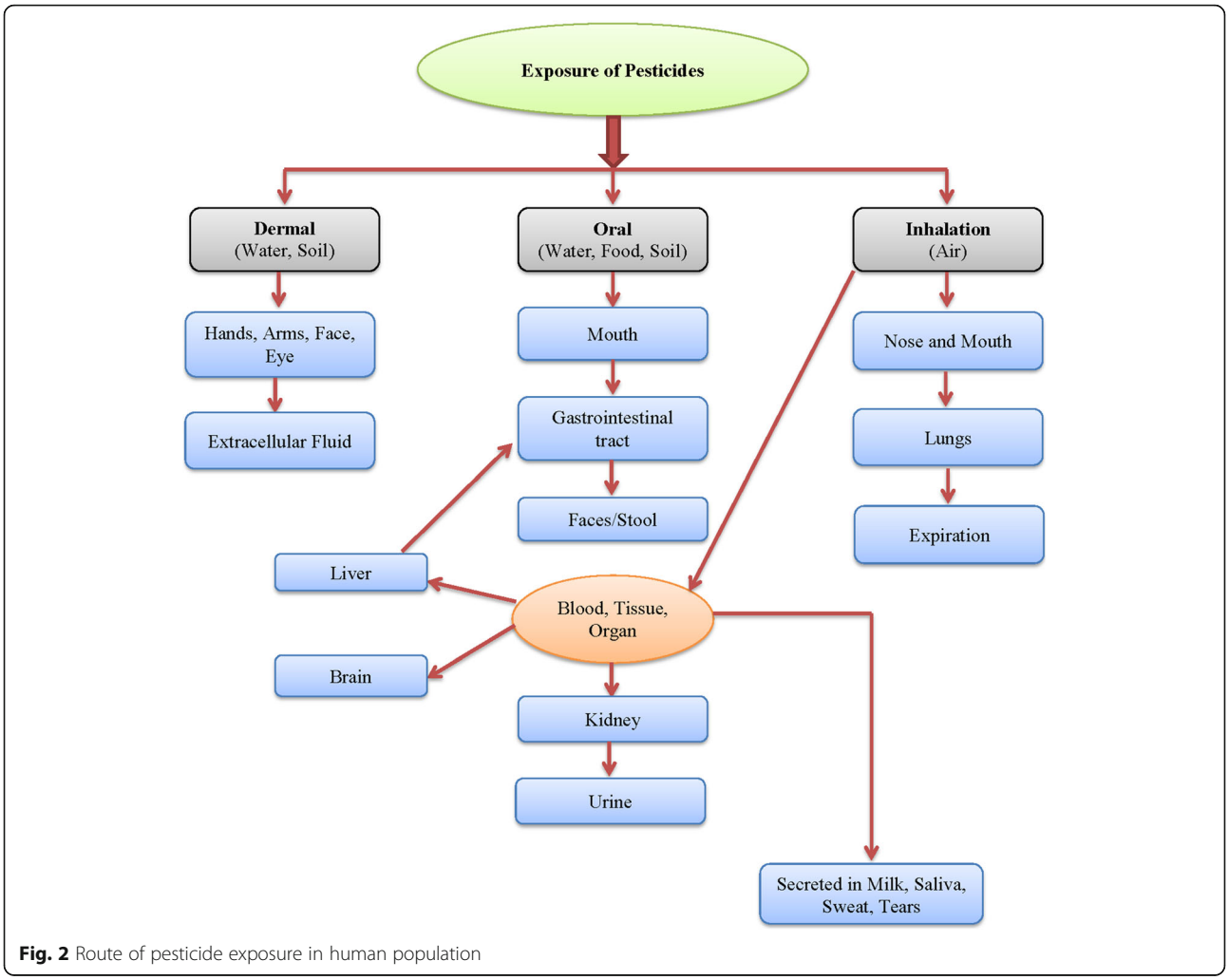


Fig. 2 Route of pesticide exposure in human population

Most pesticides including chlorpyrifos are low-molecular-weight compounds (less than 1000 Dalton) and lack epitopes, hence are not capable of eliciting an immunogenic response to produce antibodies (Cui et al. 2018). These small molecules are referred to as haptens; those could be easily altered at particular and specific positions so that a functional group could be inserted to make it more active. Further, in some cases, few derivatives of haptens can be linked with carrier proteins which could help in developing more powerful and immunogenic haptens (hapten-linker-carrier protein) that would generate specific antibodies against the chemical compound (Zeng et al. 2016; Singh et al. 2004). Design of linkers (Table 1), attachment of specific linker to a position of choice on the hapten and coupling it with the carrier are key steps for immunoassay design for pesticides. Computer-aided immunoassay design can in fact help to simulate all these critical steps such that the hypothetical immunogen with a high probability of producing required titres of antibodies in an animal model can be selected for synthesis in vitro. In this review, a computer-aided immunoassay design for chlorpyrifos has been discussed.

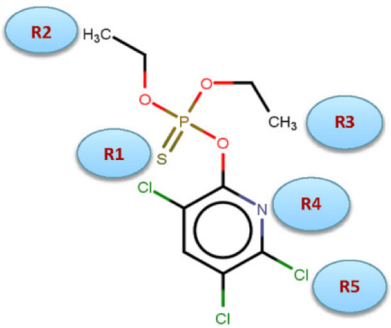
Development of an ELISA for chlorpyrifos

There are commercially available kits for CPF detection (Creative Diagnostics, Ohmicron and Millipore) along with several investigational immunoassays. These kits provide insights on positions of CPF explored for chemical modification, choice of the linker with the active functional group and carrier proteins for developing an immunogen.

Structure of CPF

Chlorpyrifos belongs to a category of organic thiophosphate. The hydrogen of the hydroxyl group of O, O-diethyl hydrogen phosphorothioate usually gets changed with 3, 5, 6-trichloropyridin-2-yl group (Koshlukova and Reed 2014) (Fig. 3a). The molecular structure of CPF lacks active functional groups, viz. –COOH, –NH2, –OH, –SH2 etc.; therefore, it cannot be conjugated with the carrier in the process of immunogen preparation as

Table 1 Designed linkers to add immunogenicity to chlorpyrifos based on available literature



S.No.	Derivatives	Reference
1.	R2–NH $(CH_2)_n$COOH	(Edward et al. 1993; Cho et al. 2002; Brun et al. 2005),
2.	R5–S$(CH_2)_n$ COOH	(Goel 2013; Manclus et al. 1994; Manclus 1996; Brun et al. 2005; Alcocer et al. 2000; Sharma and Kocher 2013; Manclus et al. 1996)
3.	R5– O$(CH_2)_n$CONH	(Lawruk et al. 1996)
4.	R4– OCNH$(CH_2)_n$ COOH	(Alcocer et al. 2000; Manclus et al. 1996)
5.	R1←O	(Manclus et al. 1996)
6.	R3–CH2COCOOH	(Jones et al. 2014)
7.	R3-CH3N(CH2)nCOOH	(Alcocer et al. 2000)
8.	R4–NH(CH_2)n-OH	(Brun et al. 2005)
9.	R3–NH(CH2)n COOH	(Hua et al. 2010; Kim et al. 2011; Liu et al. 2011; Qian et al. 2009; Wang et al. 2007; Liu et al. 2016; Maftouh et al. 2017)
10.	R3–CH3N(CH2)n COOH	(Kim et al. 2011)
11.	R3-NHCH2C6H6COOH	(Kim et al. 2011)

its structural features could easily get affected by the carrier protein micro-environment (Song et al. 2010). It necessitates CPF to be redesigned with the appropriate linker at specific sites in such a manner that it maintains the original molecular structure while making the hapten exposed on the surface of the carrier. The reason behind this phenomenon is that a molecule against which antibodies are to be made should be exposed, and in the same manner, a hapten should be easily available to the immunized cells of an organism in order to activate cells like T cell and B cell which in turn will provide antibodies which are highly specific towards the antigen.

Prospective sites for linker attachment

Primarily, two positions on chlorpyrifos have been dedicated for the site of attachment of linkers; first, one is at the 6th position on the aromatic ring and the other at the thiophosphate group by replacing o-ethyl in conjugation to a compatible linker while keeping the pyridyl ring undisturbed (Goel 2013). Manclus et al. (1994),

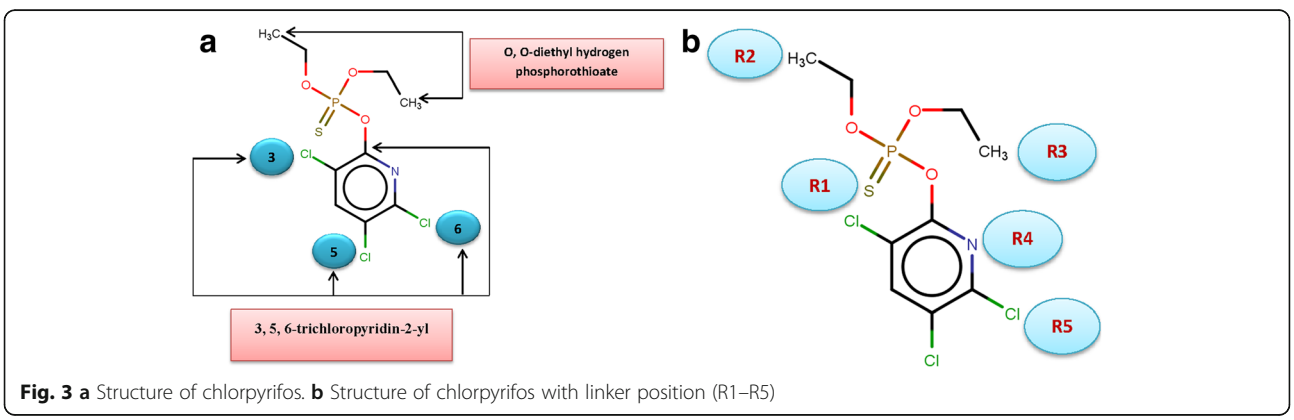


Fig. 3 a Structure of chlorpyrifos. **b** Structure of chlorpyrifos with linker position (R1–R5)

additionally, in their study, modified the pyridyl moiety at the 3rd and 5th chlorine positions; however, these modifications failed to yield any significant antibody titre by the designed immunogen (Manclus et al. 1994). Hence, there are five prospective positions for the substitution of linkers, viz. sulphur (R1) and o-ethyl (R2 and R3) in thiophosphate groups, nitrogen and 6th chlorine in the pyridyl ring (Fig. 3b).

CPF linkers

In the past, various groups have attempted the construction of immunoassay kits in different matrices (Table 2). Edward (1993) while developing an immunoassay kit for grains has replaced o-ethyl at the thiophosphate moiety with $NH(CH_2)_nCOOH$ in the CPF structure resulting in higher sensitivity and found it to be precisely more accurate than earlier monoclonal assays (Edward et al. 1993). Manclus (1994) used two kinds of linkers $NH(CH_2)_nCOOH$ and $S(CH_2)_nCOOH$ on CPF; those could produce antibodies with high specificity and sensitivity in water samples for CPF detection. This study highlighted that the highly specific antibodies should be based on specific features which are unique to the particular antigen and functional groups of the CPF apart from modification at the structural sites of the hapten (Wang et al. 2015). Manclus and Montoya (1996) designed two types of haptens, depending upon the attachment of the spacer arm, viz. the aromatic ring and thiophosphate group. The aromatic ring was altered by the introduction of different lengths of alkyl mercapto acid ($S(CH_2)_nCOOH$), chlorpyrifos oxon or chlorpyrifos methyl by the addition of an amino acid at the spacer arm. However, any modification of the alkyl mercapto acid through the aromatic ring resulted in better affinity and high specificity. Moreover, they also prepared haptens by the addition of alkyl ω-amino acids $(–CH2)_n$ of different length as ester or amide linkage of thiophosphate group and also a constructed a hapten with phenyl (C_6H_5) instead of a pyridyl aromatic ring among which (o-ethyl O-(3,5,6-trichloro-2-pyridyl) N-(5-carboxyethyl)-phosphoramidothioate and O-ethyl O-(3,5,6-trichloro-2-pyridyl) N-(5-carboxypentyl) phosphoramidothioate) resulted in increased specificity and sensitivity (Manclus 1996). Lawruk (1996) replaced chlorine at the 6th position with $CO(CH_2)_4CONH$ of pyridyl ring. This alteration allowed the selective nature of antibodies which are called monoclonal antibodies as they for chlorpyrifos detection in the different sort of products whether they are degrading or other pesticides of a different category like organophosphates (Lawruk et al. 1996). In a patent obtained by Jones et al (1997), $–(CH_2)_2COOH$ was added at the thiophosphate moiety and antibodies generated produced a high degree of specificity. Chemical substitution of S with O also played a significant part in the formation of antibody-binding sites (Reynoso et al. 2019).

Cho and co-workers published in their research paper which got published in the year 2002 used $NH(CH_2)_nCOOH$, CH_3N $(CH_2)_3COOH$ and $NHCO(CH_2)_4COOH$ as linkers to synthesize immunogens. $NH(CH_2)_nCOOH$ was noted to be successful in producing antibodies in the animal model after coupling with carrier protein (Cho et al. 2002). Brun (2005) designed five hapten derivatives by exploring two sites of the CPF structure for the addition of linkers: the pyridyl ring and the thiophosphate moiety. On the pyridyl ring, –Cl was replaced by $S(CH_2)_nCOOH$ by nucleophilic substitution reaction, and on the thiophosphate moiety, $NH(CH_2)_nCOOH$ was introduced; thus, it was proven to be highly specific antibodies for other organophosphorus compounds (Brun et al. 2005). Alococer (2000), Goel (2013) and Sharma & Kocher, (2013), in similar studies, used linkers $S(CH_2)_nCOOH$ to get antibodies with huge sensitivity and selectivity (Goel 2013; Alcocer et al. 2000; Sharma and Kocher 2013). In order to make an ELISA kit for organophosphorus pesticides like chlorpyrifos, the point to keep in mind is the hapten to be conjugated with the aromatic hydrophobic ring coupled to pesticides. When you follow this procedure, various groups successfully designed haptens by the addition of the linker to the thiophosphate moiety by NH $(CH_2)_3COOH$ of the target compound and the assay had great sensitivity (Wang et al. 2018; Cho et al. 2002; Hua et al. 2010; Kim et al. 2011; Liu et al. 2011; Maftouh et al. 2019; Qian et al. 2009).

The existing instances suggest that selecting different modification sites for hapten preparation would produce several artificial immunogens having different affinity and specificity. The immunogen maintaining the most complete structure is likely to generate efficacious antibodies. The hapten derivative in the coating antigen should share close structural similarity with CPF to make the ELISA kit with good selectivity and specificity.

Hapten derivative synthesis

Practically, it is feasible to synthesize the hapten derivatives from existing hapten, its intermediate or metabolites. Majority of haptens for OP chemicals, such as chlorpyrifos, parathion, paraoxon and phosalone can be formed from the relevant intermediates, metabolites and raw materials (Kim et al. 2003; Shim et al. 2008; Wang et al. 2017). Among the above three methods, re-synthesis is the most tedious method as it involves multi-step reactions, but is advantageous for the appropriate site as well as linking spacer for hapten modification and coupling with the carrier, respectively, which have the higher probability of producing antibodies which are higher in affinity and specific from the

Table 2 CPF detection kits, types of linkers, position of the linkers, methods of conjugation and type of antibody produced in different matrix

S. no.	Pesticides	Matrix	Linkers	Linker position	Method of conjugation	Carrier protein	Antibody	LOD	I50	Reference
1	Chlorpyrifos-methyl	Grain samples	$NH(CH2)_nCOOH$ (n = 2)	Thiophosphate moiety	Active ester method	OVA, HRP	PAb	0.02 ppm	NA	(Edward et al. 1993)
2	Chlorpyrifos	Agricultural crop	$S(CH2)_nCOOH$,(n = 2) $NH(CH2)_nCOOH$ (n = 2)	6th and 1st position of aromatic ring	Active ester method	BSA, OVA	MAb	1.3 ng/ml	33 nM	(Manclus et al. 1994)
3	Chlorpyrifos and its major metabolite 3,5,6-trichloro-2-pyridinol	water samples	$S(CH2)_nCOOH$ (n = 2) $NH(CH2)_nCOOH$ (n = 3)	6 position of the aromatic ring and thiophosphate moiety	Active ester method	BSA, OVA	MAb	6 ng/ml (chlorpyrifos) 0.034 ng /ml (TCP)	22 ng/ml (chlorpyrifos) 0.2 ng/ml (TCP)	(Manclus 1996)
5	Chlorpyrifos	Water	$O(CH2)_nCOOH$ (n = 3) OCH2COOH, $NH(CH2)_nCOOH$ (n = 2) N-C (1 carbon)	Thiophosphate moiety	Active ester method	OVA, HRP	MAb	0.10–0.14 μg/L	0.8-1.0 nM	(Manclus et al. 1996)
6	Chlorpyrifos	Water	$O(CH2)_nCONH$ (n = 4)	6^{th} position of aromatic ring	NA	HRP	MAb	0.1ng/mL	NA	(Lawruk et al. 1996)
7	Immunological detection of organophosphate (patent)	Environmental sample or biological product such as horticultural produce or foods	OCH2COOH	Thiophosphate moiety	Active ester method	OVA, BSA	MAb, PAb,RAB	NA	20 ng/ml	(Jones et al. 2014)
8	Organophosphorus pesticides (chlorpyrifos methyl, malathion, fenitrothion etc.)	NA	[(CH3O)2-P(S)-O-] (n = 4)	Thiophosphate moiety	Active ester method	BSA	PAb	NA	3 μg/ml	(Banks and Hernandez 2003)
9	O,O-Diethyl phosphorothionate and phosphorothionothiolate organophosphorus pesticides	NA	$(CH2)_nCOOH$ (n = 3)	4^{th} position of target ring structure.	Active ester method	BSA, KLH	PAb	1.6×10^{-3} μg/ml	5 ng/mL	(Johnson et al. 1998)
10	Chlorpyrifos-ethyl	NA	$S(CH2)_nCOOH$ (n = 2)	6^{th} position of the aromatic ring	Active ester method	KLH, OVA	PAb, MAb,RAb	6,6,7 ng/well	2200,4200, 1600 ng/mL	(Alcocer et al. 2000)
11	Chlorpyrifos	NA	$NH(CH_2)_nCOOH$ (n = 5) $CH3N(CH_2)_nCOOH$ (n = 3) $NHCO(CH_2)_nCOOH$ (n = 4)	Thiophosphate moiety and 3,4,5 position of the aromatic ring	Active ester method	KLH, OVA, HRP	PAb	0.1 ng/mL	20 ng/mL	(Cho et al. 2002)
12	Chlorpyrifos	Olive oil	$NH(CH2)_nCOOH$ (n = 5) $NH(Ch2)_nOH$ (n = 3) CH2COOH	Thiophosphate moiety and 6^{th} position of aromatic ring	Active ester method	HRP, OVA, BSA	PAb	0.3 ng/L, 0.07 ng/L	271 ng/L, 7 ng/L	(Brun et al. 2005)
13	Chlorpyrifos	NA	$NH(CH2)_nCOOH$ (n = 3)	Thiophosphate moiety	Active ester method	BSA, HRP	PAb	NA	0.582 μg/mL	(Wang et al. 2007)

Table 2 CPF detection kits, types of linkers, position of the linkers, methods of conjugation and type of antibody produced in different matrix *(Continued)*

S. no.	Pesticides	Matrix	Linkers	Linker position	Method of conjugation	Carrier protein	Antibody	LOD	I50	Reference
14	chlorpyrifos-methyl	Grape, Chinese cabbages, water and soil	$NH(CH_2)_nCOOH$ (n = 3)	Thiophosphate moiety	Active ester method	BSA, OVA	MAb	0.32 ng/ml	75.22 ng/ml	(Qian et al. 2009)
15	chlorpyrifos-methyl	Water samples	$NH(CH_2)_nCOOH$ (n = 3)	Thiophosphate moiety	Active ester method	BSA	MAb	0.6 μg/ml	1024.39 ng/ml	(Hua et al. 2010)
16	chlorpyrifos	Agricultural samples	$NH(CH2)_nCOOH$ (n = 5,7) $CH_3N(CH2)_nCOOH$ (n = 3)	Thiophosphate moiety	active ester method	KLH, OVA	MAb	10 and 50 ng/mL (chlorpyrifos standard and chlorpyrifos spiked)	24 ng/ml	(Kim et al. 2011)
17	Chlorpyrifos	Agricultural samples	$NH(CH2)_nCOOH$ (n = 5)	Thiophosphate moiety	Active ester method, mixed-anhydride method	OVA, BSA	MAb	0.1 ng/ml	3.3 ng/ml	(Liu et al. 2011)
18	Chlorpyrifos (review)	NA	$S(CH2)_nCOOH$ (n = 3) $NH(CH2)_nCOOH$ (n = 3)	Thiophosphate moiety and 6^{th} position of the aromatic ring	NA	NA	NA	NA	NA	(Goel 2013)
19	Chlorpyrifos	NA	$S(CH2)_nCOOH$ (n = 2)	6th position of pyridyl ring	Active ester method	BSA	NA	NA	NA	(Sharma and Kocher 2013)
20	Chlorpyrifos	Environmental and food samples	$NH(CH2)_nCOOH$ (n = 3,5)	Thiophosphate moiety	active ester method	BSA, OVA	MAb	NA	3.72ng/ml	
21	Chlorpyrifos-ethyl	Agricultural products	$NH(CH_2)_nCOOH$ (n = 3)	Thiophosphate moiety	Active ester method	BSA, HSA	PAb	NA	NA	(Maftouh et al. 2017)
21	Chlorpyrifos-ethyl and its metabolites 3,5,6-trichloro-2-pyridinol and diethylthiophosphate	Water samples	$NH(CH2)_nCOOH$ (n = 4, 5)	Thiophosphate moiety	active ester method	BSA, HSA	PAb	2.3410−2, 2.18 10−3 and 8.81 10−3 ppb for CPE, TCP and DETP respectively	CPF (651), TCP (5.61) and DETP (76.5) ppb	(Maftouh et al. 2019)

perspective of characteristic features (Song et al. 2010; Rajesh et al. 2013). Once the hapten derivative is ready, one should check its structural details with the help of different biophysical techniques.

Linking spacer

The linking between the hapten and carrier can be established by using a linking spacer that highlights the groups on the hapten which have the ability of activating the host's immune system. The basic principle for choosing the linker involves positioning the linker with a suitable length far from the functional groups (Bellemjid et al. 2018; Ning et al. 2018). Linkers, those that are too short, would likely change hapten property due to steric hindrance while linkers with too long spacers would likely be subjected to folding (Mercader et al. 2020; Song et al. 2010). Kim (2003) keeps the fact that approximately optimal spacer arm contains 4–8 carbon atoms (Kim et al. 2003). For instance, in order to produce the different derivatives of hapten with CPF, Manclus (1996) utilized a spacer arm with a five-carbon chain, while (Manclus et al. 1996) Cho (2002) used the spacer arm with only two carbons in length (Cho et al. 2002). The spacer arm shall not be comprised of few groups like the aromatic ring, conjugated double bonds or heterocyclic, etc. because in that case, the antibody would bind with the spacer arm more strongly contrary to the target analyte (Ertekin et al. 2018).

Carrier proteins

With hapten–carrier conjugates (e.g., artificial antigens), properties of the carrier should be stable, facilitate the transport of hapten and also increase the relative molecular weight of the antigen to stimulate the immunogenic reaction of the immunized organism, which can act against the haptens (Zhao et al. 2016). Once the hapten is synthesized, it should be linked to an appropriate protein through a conjugation method such as covalent bonding between functional groups of N-terminal or C-terminal of haptens and side chain of amino acids, such as lysine, aspartic, glutamic, imidazo and the phenolic functional group of histidine and the tyrosine residues, respectively, and sulphhydryl groups of cystein residues (Shim et al. 2010). Among the different carriers proteins such as bovine serum albumin (BSA), ovalbumin (OVA), keyhole limpet hemocyanin (KLH), rabbit serum albumin (RSA), human serum albumin (HSA), thyroglobulin (TG), fibrinogen and rabbit and chicken gamma globulin, BSA is a first choice for these procedures as it has highly stable physico-chemical properties, such as a good number of lysine and other free amino acids on the surface, besides that it has coupling advantage with the hapten in the solution containing organic solvents (such as pyridine, N, N-dimethylformamide) (Chen et al. 2021). KLH is excellent as it is very heterogenic in nature and heterogeneity is one of the key factors when it comes to eliciting the immune system of vertebrates, but it also has one problem as it is quite expensive. From the recent advancement in this area, it has been noticed that a synthetic polypeptide could be used as a carrier as it enhances the immunogenicity of the hapten-protein conjugate (Satija and Shalek 2014).

Computational-based approach for hapten design

Primarily, traditional hapten design (hapten and linker ligation) in immunoassay kit development follows the trial and error method (Yang et al. 2020). Therefore, all designed molecules are required to be synthesized, conjugated and used to immunize animals. Despite laborious and time-consuming series of experiments, many designed haptens fail to form stable immunogens or generate antibodies with high affinity and specificity, hence becoming inapt for developing and useful immunoassay kits (Li et al. 2015; Muldoon et al. 2000). The failures are likely to be attributed to designing haptens with low binding affinity towards carrier proteins or incomplete understanding of epitope structure and its interactions with paratope (Qaraghuli et al. 2015). Therefore, the implementation of computer-assisted molecular modeling could deliver a rational hapten design. This would aid the design of a large set of hapten derivatives both structurally and electronically most similar to the target analytes. Subsequently, computational docking and molecular dynamics simulations could further help in the selection of fewer but suitable hapten derivatives for synthesis based on better and stable binding affinity towards carrier proteins. The top-ranked stable immunogen upon synthesis in vitro is more likely to succeed in producing antibodies with high affinity and specificity in animals in contrast to the conventional approach (Xu et al. 2011).

Structural modification of pesticides

Hapten and antigen

CPF is a small molecular compound which is called a hapten. It possesses a molecular mass of 350.6 g/mol. CPF structure could be redesigned by addition of active functional groups such as –COOH, –NH and –OH at five different positions of the target compound. The studies have proposed that the redesigned structure should possess similar physicochemical properties (Gefen et al. 2015; Wang et al. 2007; Jin et al. 2005).

Another probable concept was anticipated by Xin et al (2010) that a polyclonal antiserum would be produced in the presence of an immunogenic complex conjugated with multitudinous hapten molecules against the same carrier protein (Xin et al. 2010). Edward (1993) designed the hapten molecule chlorpyrifos-methyl in which the

linker group is attached to the thiophosphate moiety of the target compound and that assay had great sensitivity (detection limit = 0.02 ppm in grain) (Edward et al. 1993).

The hapten structure plays a critical role in antibody production and quality (Kim et al. 2003). And the same is depicted in Table 2, that functional regions of CPF molecules are located at thiophosphate moiety and 6th position of the target molecule. In addition to the structure, Zhang (2010) proposed the important role of hydrophobic interaction in the antigen and antibody binding based on quantitative structure-activity relationship (QSAR) techniques along with comparative molecular field analysis (CoMFA) to predict the cross-reactivity (CR) of the PAHs in ELISA (Zhang et al. 2010). Liu et al. (2016) believed that some specific coupling sites and bonds would affect the production of antibodies. They chose the thiophosphate moiety for the attachment of the linker at the target molecule they succeeded in obtaining a monoclonal antibody with a detection limit of (LOD) 0.32 ng/ml and I_{50} of 75.22 ng/ml (Liu et al. 2016). Spier (2009) found that conformational changes of the career protein result in the internalization of the hapten within the hydrophobic pockets and thus might restrict the accessibility of the antibody and this would cause lowering the detection limit of the antibody (Spier et al. 2009). Therefore, redesigning known CPF linkers and placing them at five substitutable positions of CPF (R1-R5) generate 258 unique CPF derivatives (Table 1).

Conjugates

Apart from inducing immunogenicity by increasing the relative molecular weight, a hapten-carrier conjugate also assists hapten immobilization to develop immunoassay hapten-carrier conjugates (Ramin and Weller 2012). The method of binding is determined with the help of active groups for pesticide antigen synthesis:(1) coupling of carboxyl-containing haptens with the carrier using N-hydroxysuccinimide active ester/carbon-diimine or woodward reagent protocol (Cheng et al. 2009; Gui et al. 2006; Yan et al. 2009); (2) coupling of amino-containing haptens with the carriers in the presence of glutaraldehyde, diisocyanate, halo-nitrobenzene, diimine ester, or diazotization protocol (Pu et al. 2008; Strasser et al. 2003); (3) coupling of hydroxyl-containing haptens with the carrier through the succinic anhydride or azobenzoic acid protocol (Han et al. 2007); (4) Linking of carbonyl-containing haptens (ketone or aldehyde) with the carriers using the amino-ox-acetic acid protocol (Goel 2013). These methods are time-taking and laborious, and also, these methods may lack some features useful in developing immunoassays. Through CAMM (computer-assisted molecular modeling), we can conjugate carrier proteins with haptens by molecular docking studies. The binding affinity of hapten-carrier protein conjugates can be determined by docking score as well as free energy calculation (prime MM-GBSA method) (Sotriffer et al. 2000). Molecular docking is a technique which predicts the preferred orientation of one molecule to another to form a stable complex. It allows predicting the three-dimensional shape of a molecule such as the length, depth, dihedral angle and superimposition of a hapten with the target compound, several bonds formed in antibody-antigen interaction and electron density distribution and can contribute substantially to the understanding of recognition. It will help in choosing one of the finest haptens from the group of hypothetical immunizing haptens in order to produce immunoassays which are highly sensitive as well as selective in their function. (Xu et al. 2015). There are several docking programmes available to approximate the binding affinity of protein-ligand interaction. Glide is one such docking programme that uses an empirical scoring function—glide score. It is designed to exhaust the possibilities of separation of complexes with stout binding affinity from those with petite to no binding ability. The scoring function accounts for lipophilic-lipophilic interactions, hydrogen bond, a rotatable bond penalty, and contributions from protein-ligand coulombic and VdW energies. Furthermore, methods like molecular dynamics simulation and molecular mechanics generalized born surface area (MM/GBSA) have become the first choice of researchers in current times for different evaluations like theoretical evaluation or other methods for checking the binding of pesticides and protein. With the help of molecular dynamics (MD) simulations, one can deduce the versatility of a system. MM/GBSA takes account of various decomposition energies originating atoms or types of interactions. Conformational, interactional stability of the designed immunogen can be approximated through molecular dynamics simulation (Mohd et al. 2018; Yoshida et al. 2019). The top-ranked hapten-carrier conjugate identified to form a stable complex through CAMM can be synthesized and used for antibody generation to develop immunoassays. Accordingly, competitive indirect ELISA for fourteen O, O-diethyl organophosphorus pesticides was developed by Xu et al. (2010). CAMM is considered to be more feasible, quick and not so expensive in terms of developing an immunoassay (Xu et al. 2010). Several sensitive and specific immunoassays have been developed for the determination of metamifop (Moon et al. 2007), parathion (Shi et al. 2014), pyrethroid (Jin et al. 2017), semicarbazide (Vass et al. 2008), anti-triazine (Delaunay-Bertoncini et al. 2003) etc. with the use of CAMM methods.

Verification of hapten-protein conjugates

A quality and conjunction ratio needs to be determined after antigen preparation. The process of verification is complex and no clear statement regarding the best ratio is reported (Prechl 2017). However, although high combinations can generate desired antibody titres, it could lead to reduced binding affinity. In order to verify the quality of antigens, several techniques are being used like UV spectroscopy, mass spectrometry and SDS-PAGE (Fodey et al. 2009).

Production of antibodies

The very crucial point for an immunoassay to succeed is to produce an antibody which shows good selectivity at the same time-sensitive as well. The major component of an immunoassay is its antibody. There are different types of antibodies that are present based on the method which was followed to produce them, for example polyclonal (pAb), monoclonal (mAb) and recombinant (rAb) antibodies (Alcocer et al. 2000; Qian et al. 2009; Hongsibsong et al. 2020). Generally, pAbs are specific to many epitopes and that is why they have limited use in biological sciences, usually prepared from the blood of vertebrates (e.g. rabbits, goats and horses) all of which are immunized with hapten proteins which carry several different epitopes of which antibodies which are polyclonal in nature are produced (Leenaars and Hendriksen 2005). It has a low preparation cost and easy to develop (Lipman et al. 2005). The reason behind emulsifying the antigen and adjuvant is to make a homogenous mixture which could be used further to immunize an organism. In this manner, when the emulsifying solution is injected into an organism, the cells of the immune system like B cells and T cells start eliciting an immune response due to which a significant number of antibodies can be retrieved from the serum (Hill et al. 1994). Many pesticide immunoassays still employ polyclonal ones (Cho et al. 2002; Brun et al. 2005; Alcocer et al. 2000; Maftouh et al. 2017) as they are quite cheap and easily available in contrary to Mabs which are expensive.

As the name signifies, monoclonal antibodies (mAbs) are very specific and selective in nature in comparison to polyclonal antibodies. As they are highly specific in binding, they are being more preferred for antigen detection of any particular protein (Yan et al. 2009). Nevertheless, mAbs can be extensively used in practical testing owing to their sensitivity, specificity, simplicity and rapid detection properties. But this does not mean that it has all the solutions as it brings some limitations with it (Wang et al. 2013). Methods which are being used to prepare are just experiential and performed using non-standardized procedures and experience researcher (Cervino et al. 2008). While preparing the method, many factors need to be taken into consideration like dilution factors and conditions in which cultivation took place, for example the use of feeder cells (Jin et al. 2009). In addition to that, single hybridoma cell clones are also being screened first with the help of ELISA. A significant number of cells are being lost when this strategy is used. Hence, it is an extremely tedious and cumbersome process in itself (Zhang and Wang 2009). In general, hybridoma technology includes the immunization of mice, a combination of B cells with myeloma cells for the B cell deification. An assortment of antibody-producing hybridoma emulation in the selection of medium and scale-up antibody generation (Manclus et al. 1994; Qian et al. 2009; Manclus et al. 1996; Liu et al. 2016) produced mAbs from the hybridoma culture. In comparison to pAb production, mAb production is more difficult, laborious, time-taking and affluent.

Another method to produce antibodies is by using the recombinant antibody procedures which is more advanced in stage. Recombinant antibodies (rAbs) or their fragments (for example, scFv or Fab) are made by producing libraries of antibody gene fragments followed by phage display, ribosomal display or yeast display, from which antibodies of desired specificities and affinities tailored by site-directed mutagenesis can be selected (Alcocer et al. 2000). By using phage display methodology, single-chain variable fragment (scFv) antibodies have been generated targeting a large number of pesticides including carbaryl (He et al. 2019), methamidophos (Li et al. 2006) and fenitrothion (Luo and Xia 2012).

Method of developing an assay

Immunoassays can be developed using obtained antibodies which are based on radioactive isotopes, fluorescent enzymes and colloidal gold (Au) to quantify the measurement on the basis of a signal which is easily measurable (Yao et al. 2020). Immunoassays can be divided on the labelling materials which has been used like in radioimmunoassay (RIA) radioactive materials are being used, enzyme immunoassay (EIA) uses enzymes like HRP, fluorescence immunoassay (FIA) uses fluorescecent dyes, Chemiluminescence (CLIA) Immunosensor, molecularly imprinted technique, and surface plasma resonance sensing. Every analytic method has its own unique advantages for the detection of a sample (Boroduleva et al. 2017; Du et al. 2014; Li et al. 2009).

Radioimmunoassay

Radioimmunoassay (RIA) uses isotope-labelled and unlabelled antigens to react competitively in a stepwise formation of immune complexes. The immunoradiometric assay (IRMA) and RAST test (radioallergosorbent test) is an example of an radioimmunoassay. It included both the qualitative and quantitative tools for the detection of pesticides, antibiotics and hormones etc. (Yucra et al.

2008). I125 labels are usually applied although other isotopes such as C14 and H3 have also been used. The most important advantages of RIA are quite precision, sensitive, specific, and simple. RIA also has some of the drawbacks by using radiolabelled reagents, radiation hazards, labs require a special licence to handle radioactive material, requires special arrangement for requisition, storage of radioactive material and radioactive waste disposal (Ahmed et al. 2020). Therefore, RIA-based kits may not be advisable for CPF screening at the community level.

Fluorescence immunoassay

In a fluoroimmunoassay, the antibodies are labelled with fluorescent probes. After incubation with antigens, the antibody-antigen complexes are isolated and the fluorescent intensity is measured to quantify the target antigen. The commonly used fluorochromes in immunofluorescence are fluorescein isothiocyanate (green) and tetramethyl rhodamine isothiocyanate (red). FIA can be categorized into heterogeneous and homogeneous assays. Either heterogeneous or homogeneous assays can be performed in a competitive or non-competitive format (Sanchez-Martinez et al. 2007). Fluorescence polarization fluoroimmunoassay (FPFIA) is the most common type of assay and its principle is based on the homogenous assay format. It quantifies the change in fluorescence polarization of reaction mixtures of a fluorescent-labelled tracer, sample antigen and defined antibody. Time-resolved fluoroimmunoassay (TRFIA) is a separate group of FIA because its principles can be adapted to both heterogenous and homogenous assay formats. FIA is simple, highly sensitive and versatile. However, FIA requires expensive dedicated instrumentation, which limits its use in smaller laboratories, nonspecific binding causes quenching and fluorescence generated is changed (Zhang et al. 2020).

Chemiluminescent immunoassay

Chemiluminescent immunoassay (CLIA) is an immunoassay technique where the indicator of the analytic reaction is a luminescent molecule. In general, luminescence is the emission of visible or near-visible radiation which is generated when an electron transitions from an excited state to a ground state. The resultant potential energy in the atom gets released in the form of light. It is an alternative to radioimmunoassay as a detection principle for the determination of molecules (e.g. pesticides, proteins, and environmental contaminants). Chemiluminescent methods can be direct using luminophore markers (acridinium and ruthenium esters) or indirect using enzyme markers (alkaline phosphatase with adamantyl 1, 2-dioxetane aryl phosphate (AMPPD) substrate and horseradish peroxidase with luminol). Either method may be competitive or non-competitive (Dodeigne et al. 2000). The key advantages of chemiluminescent analytical methods reside in the wide dynamic range, high signal intensity, high specificity and rapid acquisition. The disadvantages of CLIA are represented by limited Ag detection, high costs, limited tests panel and closed analytical systems (Chen et al. 2012).

Bio-barcode immunoassays

The bio-barcode (BCA) amplification assays are utilized in the quantitative detection of small molecules, such as pesticides, veterinary drugs, and environmental toxins. The technique encompasses the use of two probes: magnetic beads coated with monoclonal antibodies for the protein and AuNP and coated with anti-target protein antibody and barcode DNA. Then, a magnetic field can be used to form a sandwich-like complex of the two probes with a test sample containing a target protein to form “magnetic microsphere-target protein-AuNP”. After dissociation of the labelled DNA barcode strands on the gold nanoprobes via dehybridization elution release, the target protein content can be determined by the selected colorimetric, fluorescence labelling, biochip or other detection methods. Signal amplification detection methods commonly used for the BCA mainly include chip methods (Feng et al. 2021), fluorescence labelling methods (Tabatabaei et al. 2020), biosensor methods (Zikos et al. 2015) and immuno-PCR methods (Dahiya and Mehta 2019). BCA has the advantages of high sensitivity, simple operation, good repeatability and good linear relationship between detection. BCA has some drawbacks; their specificity depends on the specificity of the monoclonal antibodies used in the detection system; however, the relatively high cost of commercial monoclonal antibodies and polyclonal antibodies will affect the application of this technology in actual detection. The preparation of probes takes a long time (Wang et al. 2017).

Enzyme-linked immunosorbent assay

ELISA is the preferred choice of researchers in order to detect the presence of OP pesticides. It is highly sensitive, simple to operate and can be detected at very low concentrations. “Competitive” and “non-competitive” are the two basic types of ELISA which are based on the utilization of a limited concentration of available antibodies in the sample. In competitive ELISA, the analyte and the hapten-carrier protein conjugate react with the antibodies (Ab) simultaneously, whereas in non-competitive ELISA, the Ab-binding site will be already taken, once they are exposed to analytes. Non-competitive ELISA is found to be more sensitive, has a wider scale range of detection and has higher selectivity

as compared to competitive ELISA (Fig. 4) (Cui et al. 2018).

One of the ELISA which is based on monoclonal antibodies was used for the analysis of the chlorpyrifos-methyl. The reason behind using this technique was that it is very fast for visual detection of CPF in concentrations of 5 μg/ml, and the I_{50} value of the ELISA using reflectance detection was 75.22 ng/ml, with a detection limit of 0.32 ng/ml (Qian et al. 2009). Similarly, Cho et al. developed antigen-coated ELISA (direct competitive assay and indirect competitive method) in order to detect insecticide chlorpyrifos with I_{50} of 160 ppb and a detection limit of 10 ppb. In addition, an antibody-coated ELISA was also developed, which shows an I_{50} of 20 ppb with a detection limit of 0.1 ppb (Cho et al. 2002).

Despite rapid screening of different pesticide classes by immunoassay, the sensitivity for similar types of pesticide detection is very low. The problem of the cross-reactivity of ELISA is observed in the same category of pesticides that often behave competitively to antibodies and in few cases even target the carrier protein which is being used in hapten conjugation (Manclus et al. 1996).

Generally, the cross-reactivity is checked using the analyte's curve as a standard curve to the other haptens which are quite similar to the analyte types of haptens, using analyte concentrations at 50% of the inhibition curve. Brun (2005) & Cho (2002) developed ELISA for CPF which showed 66.6%, 15.6%, 4.58% and 3.05% cross-reactivity with chlorpyrifos-methyl, bromophos-ethyl, bromophos-methyl and dichlorofenthion, respectively (Cho et al. 2002; Brun et al. 2005).

Recently developed immunoassays—advantages and disadvantages over classical tests

Immunoassay strategies are generally appropriate for water-dissolvable, polar pesticides. They can likewise be essentially quicker than certain regular techniques. Quantitative immunoassay analysis for pesticide utilized gas or liquid chromatography measures about five times the number of samples in the exact time when compared with conventional methods (Wang et al. 2019). The quick and exact nature of immunoassays depends on

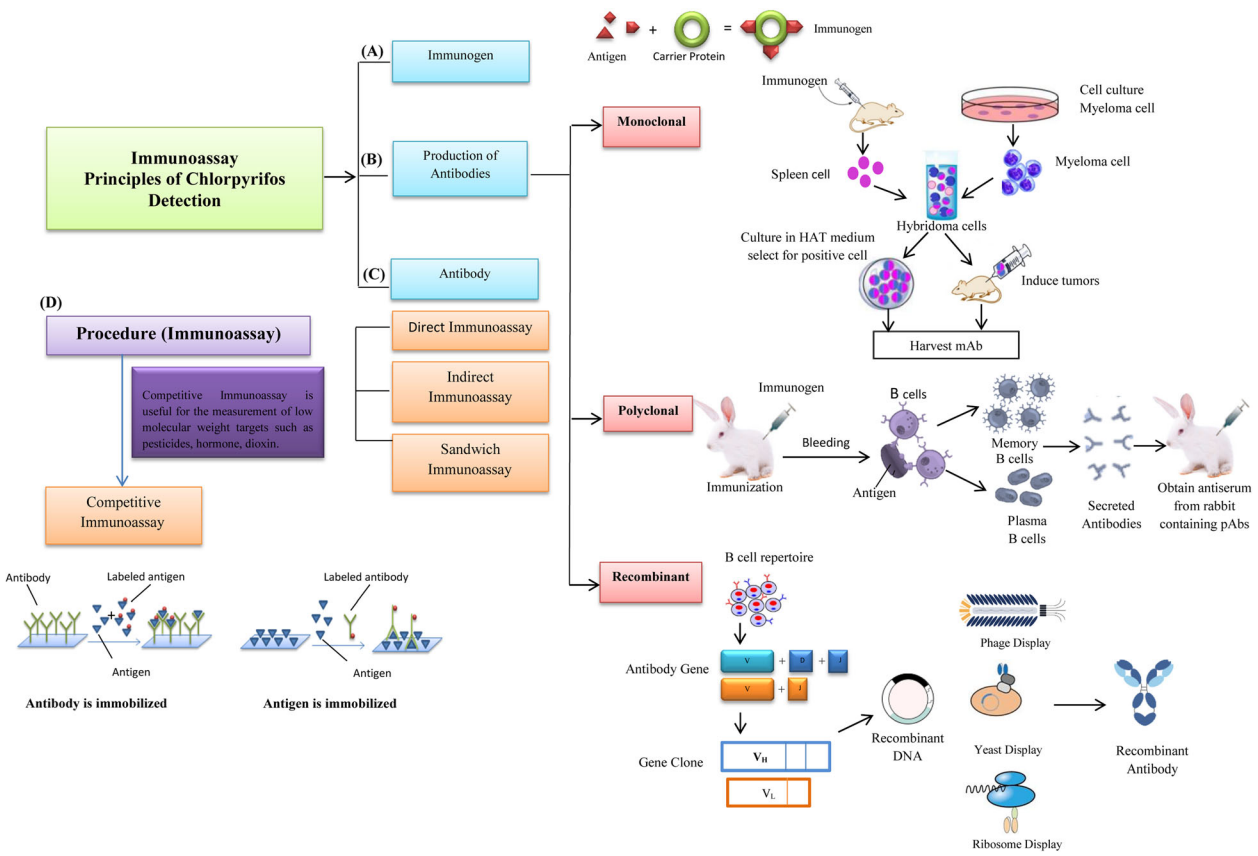


Fig. 4 **A** Small molecules can be recognized by antibody but cannot stimulate the immune system to produce antibody. **B** They must conjugate with carrier protein to form an immunogen for production of a monoclonal, polyclonal, and recombinant antibodies. **C** The antibody is a Y-shaped molecule composed of four polypeptide subunits with two identical heavy and light chains. **D** Type of immunoassay used for CPF detection

various elements. The strategy is quick as few steps can be avoided in contrast with traditional methods, for example the clean-up step can be escaped for aqueous samples (squeezes and milk), and for fruits and vegetables, and for qualitative and semi-quantitative immunoassays, the detection step may take not over 5 min. For quantitative immunoassays, the utilization of a microtiter plate which carries 96 wells can help in the identification and measurement of a big group of samples at one point in time (Jones et al. 2014). It almost takes approximately 4 to 6 h to quantify the sample. Though the immunoassay has various advantages, the use of immunoassays for monitoring pesticide residue in food is less sensitive for some compounds as compared to conventional methods, and they may have lower levels of reproducibility (Li et al. 2016). Assays which involve the use of different types of antibodies are quite selective in their approach and they are not appropriate for analysing the many residues at a time as they give false results. Hence, one can use them to study many samples in less time but they detect rarely any pesticides (Ahmed et al. 2020).

Conclusion

Recent advances in computational technologies combined with advances in molecular modelling have the potential to make important contributions to immunoassay design for CPF screening at the community level. The challenging aspect attributed to immunoassay kits for CPF has been the design of appropriate linkers that would aid the binding of CPF with carrier proteins while keeping the pesticide exposed for achieving immunogenicity in animal models. 3D molecule drawing tools and protein-ligand docking could achieve appropriate linker design and subsequent ligation with carrier proteins by exploiting knowledge of the ligand-binding pocket. Appropriate interaction among the CPF-linker-carrier protein complex (the immunogen) could greatly help in producing adequate titres of antibodies. In other words, a CPF derivative design is the most important part of an immunoassay kit for pesticides such as CPF prior to experimental validation. Moreover, as the same can be achieved in silico, a computer-aided approach offers flexibility to test all possible combinations of CPF derivatives and helps in selecting the best ones for chemical synthesis, thereby saving both time and money involved in the immunoassay design. The robust protocol for computer-aided immunoassay designs illustrated for CPF could be used as a model for other similar pesticides.

Abbreviations

CPF: Chlorpyrifos; GC: Gas chromatography; HPLC: High-performance liquid chromatography; OPs: Organophosphorus; LC-MS: Liquid chromatography-mass spectrometry; ELISA: Enzyme-linked immunosorbent assays; BSA: Bovine serum albumin; OVA: Ovalbumin; KLH: Keyhole limpet hemocyanin; RSA: Rabbit serum albumin; HSA: Human serum albumin; TG: Thyroglobulin; QSAR: Quantitative structure-activity relationship; CR: Cross-reactivity; CAMM: Computer-assisted molecular modelling; MM-GBSA: Molecular mechanics-generalized Born model and solvent accessibility method; MD: Molecular dynamics simulation; SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PAb: Polyclonal antibody; MAb: Monoclonal antibody; RAb: Recombinant antibodies; scFv: Single-chain variable fragment antibodies; RIA: Radioimmunoassay; EIA: Enzyme immunoassay; FIA: Fluorescence immunoassay; HRP: Horseradish peroxidase; TRFIA: Time-resolved fluoroimmunoassay; IRMA: Immunoradiometric assay; RAST: Radioallergosorbent test; BCA: Bio-barcode; CLIA: Chemiluminescent immunoassay

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Authors' contributions

NSK (Ph.D. Student) designed, analysed, interpreted, drafted and revised the script. DP (Scientist "C") analysed, interpreted and supervised the work. SC (Scientist "B") and PS (Ph.D. Student) analysed and revised the script. NKP (Associate Professor) interpreted, analysed and supervised the work. AKJ (Scientist "G") conceptualized and supervised the work. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare that they have no competing interests.

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