Allele specific primer designing for SNPs of ACHE gene related to oxidative stress

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Abstract: Pesticides are the chemicals which are used for agricultural and non-agricultural processes such as insecticide, herbicide, fungicide, termiticide, piscicide etc. Pesticides are generally known as chemical or biological substance designed to kill or retard the growth of pests that damage or interfere with the growth of crops, shrubs, trees, timber and other vegetation desired by humans. Pesticide formulations are complex mixtures which contain, besides the active ingredients, several other components, such as solvents, wetting and emulsifying agents, and additives (Hayes, 1991). Practically all chemical pesticides, pose long-term danger to the environment and humans through their persistence in nature and body tissue. Most of the pesticides are non-specific, and may kill life forms that are harmless or useful. Pesticides can also cause deadly diseases like cancer, Alzheimer, Parkinson, skin diseases etc.They are in direct contact with humans through air, food, or when sprayed on crops, fruits etc. They can enter in human body and mixed up. They can cause DNA damage and various type of mutations can also occur Poisonous effects of pesticides include damage to DNA such as changes or losses of nucleotic bases, and double and single-strand breakage of DNA and sister chromatid; exchanges Damaged DNA can be repaired by the different types of DNA repair mechanisms like BER, NER, mis-match repair, homologous recombinant repair etc. Pesticide formulations are complex mixtures which contain, besides the active ingredients, several other components, such as solvents, wetting and emulsifying agents, and additives. Pesticides cause oxidative stress leading to the generation of free radicals and alterations in antioxidants or free oxygen radical scavenging enzyme systems. Oxidative stress can be defined most simply as the imbalance between the production of free radicals capable of causing peroxidation of the lipid layer of cells and the body's antioxidant defence. Free radicals are defined as atoms or molecules that contain one or more unpaired electrons. The toxicity of many pesticides is associated with the production of free radicals, which are not only toxic themselves, but are also implicated in the pathophysiology of many diseases. Epidemiological studies in humans long-term exposed to a mixture of pesticides (OPs, synthetic PYRs and NMC) have reported stimulated antioxidant enzymes and lipid peroxidation in erythrocytes even in the absence of a decrease in acetylcholinesterase – AChE .

IndexTerms - ACHE, Pesticides, WASP, OPs

Introduction:
Pesticides are the substances used against pests including insects, pathogens, weeds, birds, roundworms and microbes that compete with humans for food, destroy properties or are vectors for diseases. Organophosphates are more abundtly used pesticides and it is more toxic than other classes of pesticides (Al-Salon 1994). Ops that are used now the days in agricultural practices results in an environmental pollution and number of acute and chronic poisoning events. Ops are mainly absorbed through skin.most OP exert toxicity on target or non target organs through inhibition of activity of acetylcholinesterase in nerve and muscles (Surajudiin et al 2015). The mode of exposure to ops includes the gastrointestinal and inhalatory and dermal one.(Hussain 2010).Ops mainly target acetylcholinesterase(ACHE) that hydrolyze acetylcholinesterase. The main effect of Ops involves irreversible inhibition of the activity of ACHE enzyme in blood and nervous system resulting accumulation of acetylcholine and activation of muscariniic and nicotinic receptor which may ultimately lead to death(Yshanid et al 2005). Acetylcholine esterase hydrolyzes the neurotransmitter, acetylcholine at neuromuscular junction's and brain cholinergic synapse. It is mapped to chromosome 7q22 (Ehrlich et al ., 1994). ACHE is present in molecular forms which possess same catalytic properties but they are different in their oligomeric assembly and mode of cell attachment. All enzymes are encoded by single ACHE gene. Due to alternative mRNA splicing and post translational associations of catalytic and structural subunits there is structural diversity. The quantity of Ach accumulation depends on the pesticide dose. In human beings there are two genes which encode acetylcholine hydrolyzing enymes.

Web Based Allele Specific Primer (WASP):
AS PCR is also known as amplification refractory mutation system (ARMS) . This technique is a quick and dependable genotyping protocol that requires minimal instruments found in most laboratories. It is based on the extension of primer only when its 3'-end is a perfect complement to the allele present in the input sample. Thus, if a single base polymorphism occurs, the genotyping results can be observed by simply comparing the length of PCR products. To address these difficulties a SNP database and intuitive graphical interface should be integrated with the AS primer design tool while primer destabilizing condition must be considered to ensure effectiveness of primer results. WASP is a web application constructed using Ruby on Rails Framework. The application is integrated into the local SNP database running MySQL database server. This database frequently collects public SNP information and related reference data from public SNP databases. SNPs can be detected using allele-specific PCR primers based on the 3' terminal nucleotide of a primer that corresponds to a specific SNP site. However, reliable discrimination between the alleles is not sufficient to achieve using this described method. To overcome this problem, allele specific primers with an additional base pair change within the three bases closest to the SNP site between alleles have been used. WASP offers a tool for designing AS primers for both SNPs and mutations. By integrating the database for known SNPs (using gene ID or *rs* number), this tool facilitates the awkward process of getting flanking sequences and other related information from public SNP databases. It takes into account the underlying destabilizing effect to ensure the effectiveness of designed primers. With user-friendly SVG interface, WASP intuitively presents resulting designed primers, which assist users to export or to make further adjustment to the design. This software can be freely accessed at http://bioinfo.biotec.or.th/WASP.

Material and methods:
Literature is studied for different SNP's of *ACHE* gene related to oxidative stress diseases.
Allele specific primers have been designed for these SNPs using WASP software.

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Results:
The study was carried out to design allele specific primers for various SNP's of ACHE gene.
SNP's of ACHE:
Total 6 SNP were found in different research papers which were related to diseases caused by oxidative stress.

s.no.	rs no.
1	rs17228581
2	rs7636
3	rs3757869
4	rs1799805
5	rs17228669
6	rs2396755

1. rs17228581
Sequence Size: 601
Primer Picking Parameters:
Primer Size Opt: 20 Min: 18 Max: 36
GC% Opt: 50.0 Min: 20.0 Max: 85.0
Tm Opt: 55.0 Min: 40.0 Max: 65.0
Max Tm Diff: 100.0
Max Self Complementarity: 8.0
Max 3' Complementarity: 3.0
Max PolyX in Primer: 3
Number Primer Return: 5
Mismatch at the penultimate primer position: 'Yes'
In-silico PCR Filtering: 'Yes' with Max Product Size: 4000
Mispriming Filtering: 'No'
Oligo 1 :
Wildtype Reverse Primer 5': GCCTCTCCCATGCCCAAT
Mutant Reverse Primer 5': GCCTCTCCCATGCCCAAC
Common Forward Primer 5': AATGTGGGTCTCCTGGAT
Product Size: 224
In-silico PCR Accepted.
2. rs7636
AS Primer Picking Result for rs7636
Sequence Size: 601
Primer Picking Parameters:
Primer Size Opt: 20 Min: 18 Max: 36
GC% Opt: 50.0 Min: 20.0 Max: 85.0
Tm Opt: 55.0 Min: 40.0 Max: 65.0
Max Tm Diff: 100.0
Max Self Complementarity: 8.0
Max 3' Complementarity: 3.0
Max PolyX in Primer: 3
Number Primer Return: 5
Mismatch at the penultimate primer position: 'Yes'
In-silico PCR Filtering: 'Yes' with Max Product Size: 4000
Mispriming Filtering: 'No'

Oligo 1
Wildtype Reverse Primer 5': AACTCGATCTCGTAGCCGTCG
Mutant Reverse Primer 5': AACTCGATCTCGTAGCCGTCA
Common Forward Primer 5': GGTCCTGCATTACACAGACT Product Size: 225
In-silico PCR Accepted
Oligo 2
Wildtype Reverse Primer 5': ACTCGATCTCGTAGCCGTCG Mutant Reverse Primer 5': ACTCGATCTCGTAGCCGTCA
Common Forward Primer 5': ATTACACAGACTGGCTGCAT
Product Size: 216
In-silico PCR Accepted
Oligo 3
Wildtype Reverse Primer 5': CTCGATCTCGTAGCCGTCG
Mutant Reverse Primer 5': CTCGATCTCGTAGCCGTCA
Common Forward Primer 5': CTGCATTACACAGACTGGC
Product Size: 219
In-silico PCR Accepted

Oligo 4 :
Wildtype Reverse Primer 5': TCGATCTCGTAGCCGTCG

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Mutant Reverse Primer 5': TCGATCTCGTAGCCGTCA
Common Forward Primer 5': GTGGTCCTGCATTACACAG
Product Size: 224
In-silico PCR Accepted

3. rs3757869

AS Primer Picking Result for rs3757869
Sequence Size: 601
Primer Picking Parameters:
Primer Size Opt: 20 Min: 18 Max: 36
GC% Opt: 50.0 Min: 20.0 Max: 85.0
Tm Opt: 55.0 Min: 40.0 Max: 65.0
Max Tm Diff: 100.0
Max Self Complementarity: 8.0
Max 3' Complementarity: 3.0
Max PolyX in Primer: 3
Number Primer Return: 5
Mismatch at the penultimate primer position: 'Yes'
In-silico PCR Filtering: 'Yes' with Max Product Size: 4000
Mispriming Filtering: 'No'

Oligo 1

Wildtype Forward Primer 5': CATGTGGGAGTGTGGCAGAAG
Mutant Forward Primer 5': CATGTGGGAGTGTGGCAGAAT
Common Reverse Primer 5': CCACCTCATTTCCACTAGC
Product Size: 144
In-silico PCR Accepted:

Oligo 2

Wildtype Forward Primer 5': ATGTGGGAGTGTGGCAGAAG
Mutant Forward Primer 5': ATGTGGGAGTGTGGCAGAAT
Common Reverse Primer 5': ACCTCATTTCCACTAGCGAT
Product Size: 141
In-silico PCR Accepted

Oligo 3

Wildtype Forward Primer 5': TGTGGGAGTGTGGCAGAAG
Mutant Forward Primer 5': TGTGGGAGTGTGGCAGAAT
Common Reverse Primer 5': ACCTCATTTCCACTAGCGA
Product Size: 140
In-silico PCR Accepted: There is only one PCR product in the current human genome.

Oligo 4

Wildtype Forward Primer 5': GTGGGAGTGTGGCAGAAG
Mutant Forward Primer 5': GTGGGAGTGTGGCAGAAT
Common Reverse Primer 5': ACCTCATTTCCACTAGCGA Product Size: 139
In-silico PCR Accepted.

4. rs1799805

AS Primer Picking Result for rs1799805
Sequence Size: 601
Primer Picking Parameters:
Primer Size Opt: 20 Min: 18 Max: 36
GC% Opt: 50.0 Min: 20.0 Max: 85.0
Tm Opt: 55.0 Min: 40.0 Max: 65.0
Max Tm Diff: 100.0
Max Self Complementarity: 8.0
Max 3' Complementarity: 3.0
Max PolyX in Primer: 3
Number Primer Return: 5
Mismatch at the penultimate primer position: 'Yes'
In-silico PCR Filtering: 'Yes' with Max Product Size: 4000
Mispriming Filtering: 'No'
Oligo 1 :
Wildtype Forward Primer 5': TCATCAACGCGGGAGACTTTA
Mutant Forward Primer 5': TCATCAACGCGGGAGACTTTC
Common Reverse Primer 5': GAGGGATGCAGAGAAAGAG
Product Size: 237
In-silico PCR Accepted
Oligo 2
Wildtype Forward Primer 5': CATCAACGCGGGAGACTTTA
Mutant Forward Primer 5': CATCAACGCGGGAGACTTTC
Common Reverse Primer 5': AGGGATGCAGAGAAAGAGA

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Product Size: 235
In-silico PCR Accepted
Oligo 3 :
Wildtype Forward Primer 5': ATCAACGCGGGAGACTTTA
Mutant Forward Primer 5': ATCAACGCGGGAGACTTTC
Common Reverse Primer 5': AGGGATGCAGAGAAAGAGAA
Product Size: 234
In-silico PCR Accepted

Oligo 4 :
Wildtype Forward Primer 5': TCAACGCGGGAGACTTTA
Mutant Forward Primer 5': TCAACGCGGGAGACTTTC
Common Reverse Primer 5': GAGGACTTCTGGGACTTCTG
Product Size: 151
In-silico PCR Accepted.

5. rs17228609

AS Primer Picking Result for rs17228609
Sequence Size: 601
Primer Picking Parameters:
Primer Size Opt: 20 Min: 18 Max: 36
GC% Opt: 50.0 Min: 20.0 Max: 85.0
Tm Opt: 55.0 Min: 40.0 Max: 65.0
Max Tm Diff: 100.0
Max Self Complementarity: 8.0
Max 3' Complementarity: 3.0
Max PolyX in Primer: 3
Number Primer Return: 5
Mismatch at the penultimate primer position: 'Yes'
In-silico PCR Filtering: 'Yes' with Max Product Size: 4000
Mispriming Filtering: 'No'

Oligo 1 :
Wildtype Reverse Primer 5': AGGGCTGGGCTATAACACAGAAG
Mutant Reverse Primer 5': AGGGCTGGGCTATAACACAGAAA
Common Forward Primer 5': GCTGTAACAGTTTATTGGCA

Oligo 2 :
Wildtype Reverse Primer 5': GGGCTGGGCTATAACACAGAAG
Mutant Reverse Primer 5': GGGCTGGGCTATAACACAGAAA
Common Forward Primer 5': CCGTGGCTGTAACAGTTTAT
Product Size: 158
In-silico PCR Accepted

Oligo 3 :
Wildtype Reverse Primer 5': GGCTGGGCTATAACACAGAAG
Mutant Reverse Primer 5': GGCTGGGCTATAACACAGAAA
Common Forward Primer 5': CCGTGGCTGTAACAGTTTAT
Product Size: 157
In-silico PCR Accepted

Oligo 4 :
Wildtype Reverse Primer 5': GCTGGGCTATAACACAGAAG
Mutant Reverse Primer 5': GCTGGGCTATAACACAGAAA
Common Forward Primer 5': GCTGTAACAGTTTATTGGCA
Product Size: 151
In-silico PCR Accepted

Oligo 5 :
Wildtype Reverse Primer 5': CTGGGCTATAACACAGAAG
Mutant Reverse Primer 5': CTGGGCTATAACACAGAAA
Common Forward Primer 5': GCTGTAACAGTTTATTGGCA
Product Size: 150
In-silico PCR Accepted.

6. rs2396755

AS Primer Picking Result for rs239675
Sequence Size: 601
Primer Picking Parameters:
Primer Size Opt: 20 Min: 18 Max: 36
GC% Opt: 50.0 Min: 20.0 Max: 85.0
Tm Opt: 55.0 Min: 40.0 Max: 65.0
Max Tm Diff 100.0
Max Self Complementarity: 8.0
Max 3' Complementarity: 3.0

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Max PolyX in Primer: 3
Number Primer Return: 5
Mismatch at the penultimate primer position: 'Yes'
In-silico PCR Filtering: 'Yes' with Max Product Size: 4000
Mispriming Filtering: 'No'
Oligo 1 :
Wildtype Reverse Primer 5': GGGCTGGAGGGCAAGATG
Mutant Reverse Primer 5': GGGCTGGAGGGCAAGATA
Common Forward Primer 5': AGGAGCTCCCACAATGCT
Product Size: 216

Discussion:
Genotyping is the process of determining differences in the genetic make-up (*genotype*) of an individual by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence. It reveals the alleles an individual has inherited from their parents. Genotyping is the process of determining which genetic variants an individual possesses. Genotyping can be performed through a variety of different methods, depending on the variants of interest and resources available. Single nucleotide polymorphisms (SNPs) are the most common source of human genetic variation. The potential use of SNPs for genetic mapping of complex traits, pharmacogenetics, and medical diagnostics has been much discussed. These are single base differences between DNA of different individuals. These are more suitable for genotyping markers compared to the conventional markers such as RFLP (Restriction fragment length polymorphism), AFLP (Amplified fragment length polymorphism) and SSR (Simple Sequence Repeats). SNPs are becoming genetic markers that are used in detection of risk-associated alleles linked to human diseases. Recently, massive parallel sequencing platforms such as GSFLX (Roche), Solexa (Illumina) and SOLID (Ap-plied Bios stems) have significantly reduced the cost of high throughout sequencing. A large variety of techniques for high-throughput SNP genotyping have also been developed using Taqman, Amplifluor, genome re-sequencing, and SNP arrays. These techniques are expensive and time consuming and require specialized equipments. The original SNP genotyping methods—DNA sequencing and PCR-RFLP—are laborious and expensive because they require multiple steps including size separation. AS-PCR (Allele-specific PCR) is widely used for low-throughput applications in research.
Our aim was to design allele specific primers for *ACHE* gene with WASP software. A total of 10 SNPs were selected for allele specific primers and were designed by the WASP software. Very less scientific literature is available regarding the use of allele specific primer designing of *ACHE* gene for genotyping purpose. Wangkumhang *et al* in 2007 designed five allele specific primers for *C4P21D6* gene and found WASP a good tool for designing AS Primers for both SNP and Mutations. No other study is available in the literature for the comparison with our study. In conclusion, WASP proved to be a good tool for designing AS Primers for both SNP and Mutations

9.Conclusion:
The allele specific primers were designed with WASP (a web based allele specific primer) design application for a total of 10 SNPs of ACHE gene. The tool has potential in conveniently assisting scientists in getting SNP information from local SNP database.

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