

A Review of Mitochondrial SNP Determination using Allele-Specific PCR in Forensic Identification

(Suatu Tinjauan Penentuan SNP Mitokondria menggunakan PCR Khusus-Alel dalam Pengecaman Forensik)

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ABSTRACT

Allele-specific polymerase chain reaction (AS-PCR) is a rapid and cost-effective single nucleotide polymorphism (SNP) genotyping method compared to multiplex or real-time PCR. The SNPs occurring in mitochondrial DNA (mtSNPs) is the most abundant polymorphism in humans and can be used in human identification involving mass fatality incidents. Nevertheless, the application of AS-PCR has yet to be widely applicable in forensic investigations because this method requires further development due to its limitations. This review aims to relate the applications of AS-PCR, the combination of AS-PCR with other PCR techniques and improvements imposed on this technique in past studies. A narrative literature review was carried out over 70 studies related to the mtDNA, SNP, AS-PCR, and forensic DNA. The findings suggested that AS-PCR with new adaptations in technology such as incorporating additional mismatch, choosing allele-specific primer with either common forward or reverse, gel free real-time AS-PCR analysis, and usage of fluorescence probes can improve the specificity of SNP genotyping in forensic applications. This review concludes that AS-PCR and its combination with other improvised techniques can be applied in human identification to successfully analyze degraded or low quantity samples. Nevertheless, development of more SNP loci is required to implement AS-PCR in human identification to match the current gold standard STR typing method.

Keywords: Allele-specific PCR; human identification; mitochondrial DNA; single nucleotide polymorphism

ABSTRAK

Tindak balas rantai polimerase khusus alel (AS-PCR) merupakan kaedah pengecapjarian polimorfisme nukleotida tunggal (SNP) yang cepat dan murah berbanding multipleks PCR atau tindak balas berantai polimerase masa nyata. SNP berlaku dalam DNA mitokondria (mtSNPs) adalah polimorfisme yang paling banyak diperhatikan pada manusia dan boleh digunakan untuk pengecaman mangsa dari bencana. Namun demikian, kaedah PCR khusus alel masih belum dipraktikkan secara meluas dalam kajian forensik kerana beberapa had AS-PCR menyebabkan kaedah ini masih memerlukan penambahbaikan seterusnya. Oleh yang demikian, kajian ini bertujuan untuk menghubungkan beberapa aplikasi AS-PCR, gabungan AS-PCR dengan teknik lain dan penambahbaikan yang dilakukan terhadap kaedah ini daripada kajian lepas. Tinjauan naratif ini dijalankan terhadap lebih daripada 70 kajian berdasarkan kata kunci mtDNA, SNP, AS-PCR dan DNA forensik. Hasil tinjauan mencadangkan bahawa AS-PCR dengan penyesuaian baharu dalam kaedah tersebut seperti menambahkan bes ketidaksepadan dalam pencetus khusus alel, pemilihan pencetus umum daripada pencetus kehadapan atau berbalik, AS-PCR dengan analisis masa nyata bebas daripada penggunaan gel serta penggunaan prob pendarfluor boleh meningkatkan kekhususan AS-PCR terhadap aplikasi forensik. Kesimpulannya, AS-PCR dengan gabungan beberapa teknik penambahbaikan boleh digunakan untuk menganalisis sampel manusia terurai atau berkuantiti DNA rendah daripada tempat kejadian. Namun demikian, pembangunan lebih banyak lokus SNP harus dilakukan untuk mempraktikkan kaedah AS-PCR dalam pengecaman manusia supaya kaedah ini sepadan dengan pengecapjarian STR semasa.

Kata kunci: DNA mitokondria; PCR khusus alel; pengecaman manusia; polimorfisme nukleotida tunggal

INTRODUCTION

Human dead bodies retained from mass disasters were found in pieces or burned beyond recognition. The body parts were separated from one another, and the remains co-mingled causing human identification using DNA techniques cumbersome. For instance, identification of the victims during a terrorist attack on 11 September 2001 at the World Trade Center involved application of innovative DNA typing techniques such as autosomal single nucleotide polymorphism (SNP) markers, mini short tandem repeat (STR) assays, mitochondrial DNA (mtDNA) sequencing TrueAllele and kinship analysis (Corach 2017). Nevertheless, the challenge to analyze approximately 20,000 biological human remains from the crime scene where some of the body parts were fragmented and decomposed badly had urged researchers to innovate more rapid and cost-effective DNA typing methods (Alonso et al. 2005; Kilgannon 2021).

The vast technological progress within the forensic DNA typing community had suggested mtDNA is one of the useful genomes for human identification as they refer to a group of individuals from the same maternal lineage instead of referring to individually (Hill et al. 2007). In 2016, a study that used mtDNA to identify 140-year-old unknown human skeletal remains found from an unmarked grave in Deadwood showed the unidentified skeletal remains were matched with H1 haplogroup which is found most common in western Europe ancestry. The use of mtDNA genome had proved the ability of successful DNA typing from severely decomposed samples in human identification (Ambers et al. 2016).

Moreover, mtDNA is a 5 mm histone-free circular double-stranded DNA molecule that makes the structure resist a harsh environment (Bogehagen 2012). The high copy number of mtDNA in each somatic cell contains 2 to 10 copies enables DNA typing results using mitochondrion are more likely to be obtained compared to polymorphic sites in nuclear DNA (Elson et al. 2001). Besides that, the haploid and monoclonal nature of mtDNA makes the interpretation of DNA sequencing easier (Legros et al. 2004). Mitochondrial DNA has a higher mutation rate in the control region estimated at 0.32×10^{-6} /site/year compared to 0.5×10^{-9} /site/year in nuclear DNA (Scally 2016; Sigurdardóttir et al. 2000). The high interindividual variability in hypervariable regions of mtDNA can be very useful characteristic for forensic identification purposes (Amorim, Fernandes & Taveira 2019).

SNPs are single base sequence variations and estimated to occur 1 in every 1000 bases of human genome (Sobrino, Brión & Carracedo 2005). The

mutation rate of SNPs (10^{-8} vs. 10^{-3}) approximately 100 thousand times higher than STRs enables it to be used as biallelic markers in population studies (Mo et al. 2016; Zainuddin & Goodwin 2004). Presently, STRs are used in many forensic cases due to their large number of alleles (Warshauer et al. 2014). Nevertheless, severely degraded human samples contain amplicon sizes smaller than 100 bp in length, thus the STRs are often fragmented causing amplification of PCR products to fail (Fungtammasan et al. 2015). Thus, analysis of degraded samples using biallelic SNPs is more suitable as this only requires amplification of shorter DNA segments (~40 bp at minimum). It is more sensitive for low quantity DNA from degraded samples.

There are several SNP genotyping assays based on primer extension, allele-specific hybridization, invasive cleavage, oligonucleotide ligation, real-time PCR, high-resolution melting analysis, TaqMan probes, restriction digestion and mass spectrometry (Avanus & Altinel 2017; Barua et al. 2021; Myakishev et al. 2001; Papp et al. 2003; Wu et al. 2005). The common SNP genotyping method is allele-specific PCR (AS-PCR) which has been widely used to detect point mutations in the DNA template.

AS-PCR works by using wild and mutant type primers which form a mismatch at 3' location of non-target allele that would be refractory to primer extension by Taq polymerase (Simsek & Adnan 2000). It helps find mutations contributing to disease and some SNP alleles are used as genetic markers in population studies (Ambers et al. 2016; Sobrino, Brión & Carracedo 2005). Besides that, the uses of AS-PCR are also extended to ABO blood typing, Y-chromosome haplotyping, gene mapping, paternity testing and human identification (Gayden et al. 2008; Urata et al. 2004; Ye et al. 2001).

AS-PCR allows effective discrimination of SNP from pools of samples to establish population frequencies (Salisbury et al. 2003). Therefore, this technique has been suggested for human identification in mass fatality incidents but it requires the development of more SNP loci to match the current STR typing method (Tamaki & Jeffreys 2005). Learning the underlying techniques of AS-PCR and its applications in various fields are vital to cater to the development of this method in human identification. Hence, this review aims to highlight: 1) The mechanism of Allele-specific PCR, 2) The applications of Allele-specific PCR in various fields, 3) Comparison between AS-PCR techniques, and 4) Improvements in AS-PCR from past studies.

SURVEY METHODS

This project is a full review of past papers ranging from the year 2000 to 2022. The focus of this paper is on the

applications of AS-PCR including in aid for forensic science, comparison between AS-PCR techniques and improvements imposed on this method. We systematically selected papers titled with keywords such as mitochondrial DNA, single nucleotide polymorphism, allele-specific PCR, DNA genotyping and forensic science. Our search was not refined by the impact factor of journal, authors or their affiliation.

THE MECHANISM OF ALLELE-SPECIFIC PCR

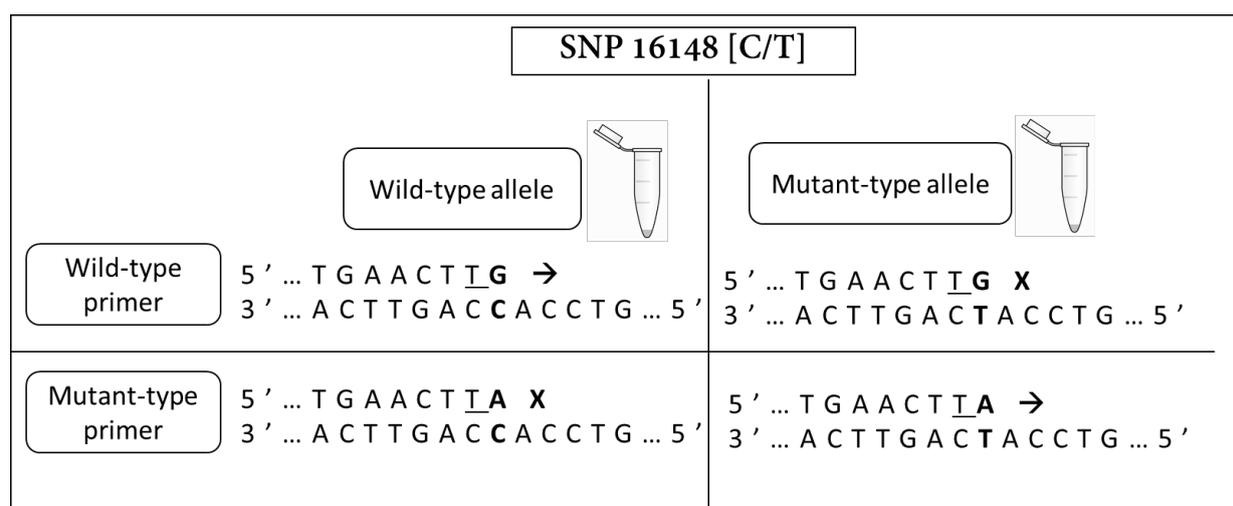
Allele-specific PCR is a technique exclusively used in DNA analysis which was discovered by C.R. Newton and his team (Gaudet et al. 2009). This technique is mainly used for SNP genotyping with the help of a refractory primer that will bind specifically to the particular allele of interest (Senafi et al. 2014). It is also named Amplification Refractory Mutation System (ARMS-PCR) due to the usage of two different primers for two different alleles. Two sets of primers are designed in which wild-type primer matches the wild allele and mutant-type primer matches the mutant allele. The modified 3' end of the primer precisely amplified with one target allele over another (Wu et al. 2005).

The primary foundation of AS-PCR is built by exonuclease activity of thermostable Taq DNA polymerase (*Thermus aquaticus*). The Taq DNA polymerase lacks a 3' to 5' proofreading activity, therefore refracting the amplification of either wild-type or mutant-type primer when the polymerase encounters a non-target allele during annealing. Besides that, the usage of 'proofreading' DNA polymerases such as *Pfu* (*Pyrococcus furiosus*) and *Pwo* (*Pyrococcus woesei*) polymerases should be avoided as it enables correcting

nucleotide incorporation errors (Špibida et al. 2017). The presence of mismatch at 3' will modify the DNA from the polymerization domain to an N-terminal 3' → 5' exonuclease domain of the polymerase to excise the misincorporated base. After removal of the mismatch, the DNA returns to polymerization domain and the polymerase resume the extension of template (Zahurancik, Klein & Suo 2014).

Furthermore, the introduction of an additional mismatch between primer and template DNA is vital in achieving the amplification (Sobrinho, Brión & Carracedo 2005). The mismatch needed to be introduced ideally at second position near the 3' end of the primer, hence it alters the annealing temperature of a particular allele and avoids cross-amplification at non-target region (Bui & Liu 2009; Simsek & Adnan 2000). Nevertheless, the reverse primer which is not modified remains the same for both alleles (Rejali, Moric & Wittwer 2018). The primer length of allele-specific primer should be between 20 to 30 nucleotides and fulfill all the ideal primer requirements. The sample illustration of underlying AS-PCR mechanism for C16148T of mtDNA is shown in (Figure 1).

After the polymerase chain reaction, the allele-specific products will undergo post-PCR detection at final to confirm the presence of amplification in either wild or mutant lane. The post-PCR detection methods can be done via electrophoresis, mass spectrometry, microarrays, fluorescence resonance energy transfer (FRET), fluorescence polarization (FP) and luminescence (Sobrinho, Brión & Carracedo 2005). Electrophoresis is the simplest and easiest way to detect locations of bands within the gel by directly staining with fluorescent intercalating dyes such as ethidium bromide and SYBR Green (Green & Sambrook 2019).



The arrow → shows allele-specific primer complement to the allele on DNA template and amplification occurred. The cross X indicates the allele specific primer does not complement to the allele and failed to amplify the template. A strong destabilizing mismatch T was introduced at the penultimate base (second position from 3' end of primer) to enhance the reaction specificity

FIGURE 1. Illustration of allele-specific PCR mechanism of primer binding to wild and mutant alleles of SNP C16148T in human mtDNA

APPLICATIONS OF ALLELE-SPECIFIC PCR

Paternity and kinship testing

AS-PCR is used in paternity testing to determine fatherhood and kinship analysis to establish familial relationships by comparing DNA fingerprint of a child, mother and father (Ma et al. 2006). The problem arises in paternity testing especially in motherless cases if the allele inherited from a father often cannot be determined reducing the probability of a genetic marker (Lee et al. 2000). In this case, mutagenically separated PCR (MS-PCR) which is an improved version of AS-PCR method can be used to determine the parental origin of alleles using DNA methylation markers (Zhao et al. 2005). The imprinted SNP locus rs22008 (A/G) was chosen as a model system to determine the obligatory genes of the parents. It is cost and time effective as the analysis can be conducted in a single tube simultaneously.

Disease genotyping and diagnosis

Furthermore, this technique is also used in the medical field for disease genotyping and diagnosis. According to World Health Organization (WHO), ischemic heart disease is the number one leading cause of natural death in the world. Therefore, researchers had used AS-PCR and successfully identified that carriers of AT genotype in rs2274907A>T were more susceptible to coronary artery disease (Jha et al. 2019). Besides, modified AS-PCR had also been developed to detect HER2 Ile655Val gene polymorphism that is associated with cancer development (Budiarto, Azamris & Desriani 2017). Furthermore, Marini et al. (2012) demonstrated the accuracy of AS-PCR in detecting absence or deletion of *SMN1* gene for routine diagnostic screening of spinal muscular atrophy. Hence, the reliability of AS-PCR as a rapid technique in diagnostic screening has been proven in many clinical studies.

Y-chromosome haplotyping

Moreover, AS-PCR is also used in Y-chromosome haplotyping to study the evolutionary process of humans from paternal lineage. Y-chromosome is passed down from a father to son without recombination. The low mutation rate in Y-chromosome retained the haplotypes, thus allowing determination of genetic profiles in population studies (Butler 2012). It is used in cases when standard autosomal DNA profiling does not give much information about the identity (Kayser 2017). For instance, Gayden et al. (2008) proposed screening

SNPs using AS-PCR within the non-recombining site of the human Y-chromosome. The analysis of over 50 SNPs successfully defined the major Y-haplogroups. The validity of the detected point mutation alleles was confirmed with restriction fragment length polymorphism (RFLP) and direct sequencing of the PCR products. Therefore, AS-PCR is suitable for detection of Y-chromosome SNPs in a single expeditious method.

Genetic fetal screening

This technique has also been used in various genetic fetal screening including sex determination, hemolytic disease of fetus and newborn (HDFN), rhesus D typing, paternally inherited genetic alteration such as cystic fibrosis and beta thalassemia (Finning et al. 2007). ARMS real-time PCR was feasible to screen beta-thalassemia mutant alleles from non-invasive prenatal testing approach using circulating cell-free fetal DNA in maternal plasma (Afzal et al. 2022). Besides that, Nasis et al. (2004) had improved the sensitivity of ASPCR to detect carriers of cystic fibrosis in cell-free fetal DNA circulating in maternal blood using Taq polymerase with no proofreading activity combined with TaqMaster PCR enhancer to prevent nonspecific amplification of wild-type allele. Thus, AS-PCR has been proved as a reliable method in genetic fetal screening by precluding pregnant women from invasive procedures.

Human identification

Furthermore, AS-PCR is also a way of DNA profiling that narrows down the victim by population to aid in disaster victim identification (Kofradi & Rebrikov 2006; Rajagopalan & Fujimura 2018). Ishar and Din (2018) had genotyped 20 mtSNPs using AS-PCR which are specific to Southeast Asia haplogroups E, M9, B4, N21, R21 and F1a1a. Besides that, DNA analysis of degraded biological samples retrieved at crime scenes often challenging to forensic scientists due to exposure of the sample in a harsh environment (Ricaud, Bellatti & Lahr 2006). Therefore, Asari et al. (2009) had successfully typed degraded and low copy number samples using AS-PCR. 18 SNPs were genotyped based on the mini-primer allele-specific amplification combined with universal reporter primers. The rate of successful DNA typing for SNP profiles is high because only 40 ng of DNA per reaction is sufficient to use in AS-PCR. This will be useful in genotyping degraded samples where the amount of genomic DNA becomes a limiting factor (Myakishev et al. 2001).

COMPARISON BETWEEN ALLELE-SPECIFIC PCR TECHNIQUES

The routine DNA genotyping involves DNA extraction, amplification, purification, electrophoresis and sequencing. Nevertheless, sequencing large pool of samples retrieved from a crime scene is expensive, time-consuming and laborious. Thus, AS-PCR has been brought forward to directly genotype mutations without the need for sequencing. Therefore, the idea of developing AS-PCR has been put forward to offer an alternative option for human identification with lower cost, easy yet robust (Muro et al. 2012). Various new methods within AS-PCR have been developed for applications in DNA analysis which could enhance performance compared to standard SNP genotyping methods (Kim, Park & Lee 2017).

The traditional genotyping assay involves PCR amplification followed by restriction fragment length polymorphisms (RFLP). Species identification using RFLP is not feasible for forensic samples in highly fragmented and deteriorated conditions because the restriction site might be absent (Avanus & Altinel 2017). Besides that, it also increases the turnaround time for analysis (Doi et al. 2004). Therefore, Nittu et al. (2021) mentioned that AS-PCR is more accurate and simple method for having the SNPs which supersedes any other PCR-based methods. This has been proved with their concurrent results of accurate species and sex identification of tiger at 56.3% ($n = 107$; 64 male and 43 female) and leopard at 38.9% ($n = 74$; 41 male and 33 female).

First, multiplex allele-specific PCR (MAS-PCR) is useful in the detection of multiple SNP sites in a gene using allele-specific primers since it can be amplified in a single tube. Based on Kim, Park and Lee (2017), their method using MAS-PCR produced precise, accurate results and successful identification of various biological samples such as blood, saliva, hair and vaginal swab. DNA amount of 60 pg was sufficient to produce results. A study using single-base primer extension method along with multiplex PCR was able to produce results as low as 0.1 ng DNA from bones, decomposed organs, and vaginal fluid contaminated with semen (Doi et al. 2004). It is a rapid and accurate method that can also be used for degraded DNA samples (Lee et al. 2013). However, these two methods involve a long process of DNA extraction, PCR amplification, gel electrophoresis and followed by sequencing. Thus, Lee et al. (2010) used a direct multiplex allele-specific primer which does not require DNA isolation and sequencing step. It was able to produce results from DNA amount as low as 60 pg

through direct characterization of samples such as blood, hair and saliva in about 70 minutes.

In addition, another method of allele-specific real-time PCR has been used in ABO blood typing does not require post-PCR manipulations and produces rapid results. A study found that 2.0 ng of DNA produced accurate genotyping and the small size of PCR amplicons needed (not longer than 107 bp) was suitable to genotype degraded DNA (Muro et al. 2012). The risk of contamination is low as there are no extensive steps. Another study by Makanga, Christianto and Inazu (2015) describes the allele-specific real-time PCR method using SYBR green which is a non-specific DNA fluorescence dye that is cheaper than fluorescence-labeled oligonucleotides. This method does not require product purification or restriction enzyme cleavage. Although this study used this method for the detection of urate 1 transporter mutation, it was also mentioned their method can be applied for genotyping other mutations. More research regarding forensics can be conducted using this method for further validation.

Minisatellite variant repeat mapping by PCR (MVR-PCR) is used to investigate allelic diversity and mutation mechanisms at the minisatellite locus (Makanga, Christianto & Inazu 2015). The internal variation provides a powerful tool to study allelic variation and stages of mutation (Suenaga & Nakamura 2004). Apart from human genotyping, a study proposed MVR-PCR is also used in identifying parasite strains (MacLeod 2004; MacLeod et al. 2001). This technique is time-effective and does not require separation methods because DNA specific to individuals is possible to be extracted in case of mixed samples. Degraded samples and samples as little as 1 ng were enough to produce reliable results (Tamaki & Jeffreys 2005).

In addition, an approach of using competitive duplex AS-PCR (ARMS-DCA) could successfully detect DNA mixtures and only a short template is required for amplification (50-70 nt). Mitochondrial SNPs has become of interest in forensics due to its stable lineage and haplogroups compared to STRs (Huszar et al. 2022). Although it does not provide personal identification, it will be useful in cases where close relatives are not available for comparison. Niederstätter and Parson (2009) proposed ARMS-DCA could be a valuable tool for large volume studies targeting a small number of SNPs. This gives an edge for analyzing forensic samples since most samples would be degraded or found in very little quantities. It is a high-throughput method that does not require post-PCR manipulations, time-saving and cost-effective.

Furthermore, classification of mtDNA variants using criteria reported by researchers is vital before genotype using AS-PCR (Richards et al. 2015). Polymorphism is described as variant with a frequency above 1%. Frequency of variants in differential racial populations is used to assess its potential pathogenicity. This can be done by assessing Mitomap (<https://mitomap.org/foswiki/bin/view/MITOMAP/WebHome>) which main database that contains over 275 mtDNA variants and haplotype information. The frequency of variants in a population and classification into its haplogroups (<http://www.phyloree.org/tree/index.htm>) provide a useful feature in interpreting mtDNA variants for AS-PCR (van Oven 2015).

Based on these insights, we may conclude that AS-PCR has a wide prospect in forensic application. Each different technique in AS-PCR plays a different role but some carry a few limitations which might downgrade the method. However, these limitations can be prevented by improvising the techniques according to high throughput technologies. For instance, the use of direct PCR eliminates DNA extraction step; application of quantitative AS-PCR does not require post-PCR detection; and using allele-specific primers producing different lengths of product in multiplex PCR could be

considered apart from the standard protocol of AS-PCR. (Gaudet et al. 2009; Imyanitov et al. 2002; Niederstätter & Parson 2009). Last but not least, considerable improvements could be made to apply AS-PCR in human identification.

IMPROVEMENTS IN ALLELE-SPECIFIC PCR [2010-2021]

The SNP genotyping methods are categorized into primer extension, hybridization, ligation, and enzymatic cleavage. Nevertheless, the standard AS-PCR based on primer extension is widely preferred by many researchers due to its simplicity and cost-effectiveness. It also became a reliable and robust technique for SNP genotyping involving human mitochondrial DNA. Besides that, many studies had used improvised versions of AS-PCR to detect point mutations for disease diagnosis, crops production and human populations. There are hundreds of studies conducted using AS-PCR. Nevertheless, only distinctive studies related to modifications from standard AS-PCR method were chosen to be reported in this review. This section examines the several improvements applied to AS-PCR from the year 2010 to 2022 and the implications of this on future prospects. In Table 1, we present some of the improvements made to AS-PCR technique to enhance the SNP genotyping.

TABLE 1. Improvements made to AS-PCR techniques from the year 2010 to 2022

Year	Study by	Purpose of study	Improvements	Advantages
2010	Lee et al. (2010)	Detection of SNP 261, 526 and 803 for ABO blood typing	Multiples AS-PCR has been developed to identify A, B, O01, O02, O03, and cis-AB01 alleles in around 70 min from sample collection to electropherogram	<ul style="list-style-type: none"> • Time and cost-efficiency • Convenience • Reduced contamination during analysis
2012	Liu et al. (2012)	Primer specificity for rapeseed (<i>Brassica napus</i> L.) and sesame (<i>Sesamum indicum</i>)	The addition of artificial mismatches within three bases from 3' end of AS-PCR primers will increase the discrimination power in SNP genotyping. SNPs (A/T) which contained CA mismatches at third nucleotide position from 3' end of primers had the highest allelic specification	<ul style="list-style-type: none"> • Choosing additional mismatches will increase the primer specificity and yield a good DNA result
2012	Muro et al. (2012)	Detection of SNP 261, 796, 803 in ABO gene and determination of 6 ABO genotypes	Developed allele-specific primers and real-time PCR for ABO genotyping	<ul style="list-style-type: none"> • This method required less than 2 hours • Requires only 2.0 ng of DNA

2013	Taira et al. (2013)	Detection of 8 SNP loci using droplet AS-PCR	The collected samples were pretreated with proteinase K and directly amplified in droplet AS-PCR without DNA extraction. Specific PCR amplification for all loci was achieved. All the genotypes were determined within 9 minutes	<ul style="list-style-type: none"> • Detection limit for droplet AS-PCR as low as 0.1 - 5.0% by dilution • Provide ultra-rapid genotyping • Reduction of total analysis time
2013	Fonseca et al. (2013)	Detection of bovine κ -casein double polymorphism Thr136Ile/Ala148Asp. Genetics	The specific genotyping method was achieved by choosing lower annealing temperatures for the allele-specific primers	<ul style="list-style-type: none"> • Offers easy, cost-efficient, highly sensitive and specific detection
2014	Medrano & de Oliveira (2014)	Genotype SNP rs9550621 and rs3751385	Application of tetra primer amplification refractory mutation system PCR (ARMS-PCR) using four primers in a single PCR	<ul style="list-style-type: none"> • Allows study of SNPs in fast, reliable and low cost way
2015	Smith & Maughan (2015)	Development of KASPar TM	A cost-effective SNP genotyping method was proposed based on competitive AS-PCR of target amplification and fluorescence genotyping (KASPar TM) using FRET capable plate reader or Fluidigm's dynamic array. A single 96.96 Fluidigm IFC can produce 9,2016 genotypic data	<ul style="list-style-type: none"> • A strong allele-specific signal is released from fluorophore when it was detached from the quencher • Inexpensive
2015	Makanga, Christiano & Inazu (2015)	Detecting two main mutations in human urate transporter 1: W258X and E90H	Allele-specific real-time PCR can detect mutations without the need of gel electrophoresis	<ul style="list-style-type: none"> • Simple, rapid and relatively inexpensive
2016	Moran et al. (2016)	Detection of TCF7L2 gene variants causing Type 2 diabetes mellitus	Standardized AS-PCR for associated mutations of rs7903146, rs12255372 and DG10S478	<ul style="list-style-type: none"> • Mutations were detected directly on the gel • The use of expensive instrumentation and reagents not needed
2017	Budiarto, Azamris & Desriani (2017)	Detection of HER2 Ile655Val gene polymorphism	Betaine treatment and modifying AS-PCR program (reducing the template concentration by half, increasing denaturation at 96°C, annealing time of 40s at first 10 cycles) could reduce genotyping errors of this technique	<ul style="list-style-type: none"> • Eliminate locus and allele drop out
2018	Alyethodi et al. (2018)	Genotype bovine leukocyte adhesion deficiency (BLAD) in cattle	The study used thermostable strand displacement polymerase (SD) replacing Taq polymerase in tetra primer- ARMS PCR	<ul style="list-style-type: none"> • All amplicons were generated by 25 cycles whereas Taq polymerase needed a minimum of 35 cycles • The addition of PCR enhancer (dimethyl sulfoxide) is not needed

2018	Majeed et al. (2018)	Development of kompetitive AS-PCR	Kompetitive AS-PCR (KASP) is a gel free single plex SNP genotyping platform based on allele-specific oligo extension and fluorescence resonance energy for signal generation	<ul style="list-style-type: none"> • Does not require post PCR handling • The PCR product directly genotyped in computer scoring software
2018	Ishar & Din (2018)	Development of mtDNA markers for human identification	Optimized AS-PCR using a two-step PCR approach with internal controls to be used in forensic cases	<ul style="list-style-type: none"> • Direct detection of wild or mutant allele on gel • Sequencing of PCR product not needed • Cost and time effective • This DNA analysis technique can be carried out at crime scene site
2019	Mastrantonio et al. (2019)	Investigate the occurrence of paternal leakage and mtDNA heteroplasmy in <i>R. sanguineus s.l.</i> ticks	A multiplex AS-PCR assay was developed targeting a fragment of 12S rRNA region of mtDNA	<ul style="list-style-type: none"> • Enable detection of paternal leakage and mtDNA heteroplasmy • Eliminate the need for sequencing
2019	Lefever et al. (2019)	Detection relevant somatic mutations on cancer cell lines	The study used basic AS-PCR combine with DNA-binding dye based qPCR technology. The double-mismatch allele-specific qPCR (DMAS-qPCR)	<ul style="list-style-type: none"> • High analytical sensitivity and specificity • Easy to use • Does not require labelled probes
2020	Chubarov et al. (2020)	Detection of cancer development gene Kirsten rat sarcoma (KRAS) mutation using phosphoryl guanidine modifies primers	The study used a novel modified oligonucleotide with internucleotide phosphates reshaped 1,3-dimethyl-2-imino-imidazolidine moieties (phosphoryl guanidine groups) as primers and blockers in AS-PCR method	<ul style="list-style-type: none"> • Increased the discrimination power between wild-type and mutated DNA • Phosphoryl guanidine increases primer specificity
2021	Wijaya et al. (2021)	Screening homozygous <i>SMN1</i> deletions causing spinal muscular atrophy (SMA) using AS-PCR based on DNA melting peak analysis	AS-PCR technique combination with melting peak analyses successfully distinguished patients with and without <i>SMN1</i> . The dried blood samples collected using FTA elute cards had preserved the DNA for good amplification	<ul style="list-style-type: none"> • Biosafe collection, transfer and storage of FTA cards • Direct PCR approach after purification, thus eliminating the need for DNA extraction • Prevents contamination • Preclusion of fluorescent probes leading to low PCR cost fast and high-throughput screening for <i>SMN1</i> deletions
2022	Kalendar et al. (2022)	SNP genotyping in barley genes <i>HvSAP16</i> and <i>HvSAP8</i>	A modified and improved version of allele-specific quantitative PCR (ASQ) based on fluorescence resonance energy transfer (FRET). The fluorophores and quencher are in separate complementary oligonucleotides	<ul style="list-style-type: none"> • Cheaper than Amplifluor and KASP • Provide clear and effective measurement of the fluorescence emittance • Low signal background-noise

CONCLUSION

There are many improvements implemented in allele-specific PCR from past 10 years in various aspects such as detection of mutation for disease diagnosis, paternity testing, ABO blood typing, sex determination, forensic investigation, genetic fetal screening. The combination of general AS-PCR combined with other modifications could increase the discrimination power of this method. Thus, this review focussed to identify the ability of AS-PCR to be incorporated in human identification involving mass fatality incidents.

As for now, only Ishar and Din (2018) had used a two-step AS-PCR approach to optimize mtDNA SNPs for human identification. Nevertheless, this study was only able to optimize 20 mtSNPs out of 30 mtSNPs due to the failure of allele-specific primers to detect the wild-type or mutant-type alleles specifically. The ideas from past studies as highlighted in this review such as designing allele-specific primer with incorporation of additional mismatch; choosing the AS primer that works best with either common forward or reverse primer; using gel-free real-time AS-PCR analysis; and usage of fluorescence probes could be taken into account to improve the method. Hence, AS-PCR needs to cater new adaptations in technology to improve the specificity of SNP genotyping.

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