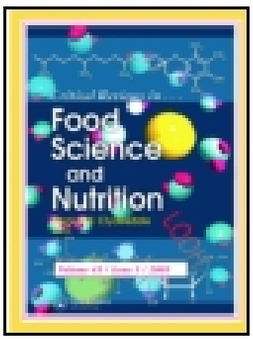

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REVIEW



# Authentication of Halal and Kosher meat and meat products: Analytical approaches, current progresses and future prospects

M. A. Motalib Hossain[a], Syed Muhammad Kamal Uddin[a], Sharmin Sultana[a], Yasmin Abdul Wahab[a], Suresh Sagadevan[a], Mohd Rafie Johan[a], and Md. Eaqub Ali[a,b]

[a]Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, Kuala Lumpur, Malaysia; [b]Centre for Research in Biotechnology for Agriculture (CEBAR), University of Malaya, Kuala Lumpur, Malaysia

**ABSTRACT**

Meat and meat products are widely consumed worldwide as a source of high-quality proteins, essential amino acids, vitamins, and necessary minerals. The acceptability of Halal and Kosher meat products relies not only on the species origin but also on the manner of slaughtering of animals. Both Islam and Judaism have their own dietary laws in their holy books regarding acceptance and forbiddance of dietary items particularly meat and meat products. They also include many strictures to follow for ritual cleanliness of foods. Since the authenticity of Halal and Kosher food created increased concerns among consumers, the integrity of Halal and Kosher meat and meat products must be assured so that consumers can be accomplished with the originality of products. There is an increasing demand for reliable and sensitive techniques for the authentication of various Halal and Kosher meat products. This up-to-date review intends to provide an updated and extensive overview critically on the present situation, progress, and challenges of analytical techniques to authenticate animal species in meat items. It also addresses slaughtering procedure with brief discussion on Halal and Kosher laws with a view to creating consumer awareness against fraudulent practices. The available methods are schematically presented, and their salient features are comparatively elucidated in tables. Potential future technologies are predicted, and probable challenges are summarized. Overall, the present review article possesses substantial merits to be served as a reference guide in the field of academia and industry for the preparation/processing and identification of Halal and Kosher meat and meat products as well as may act as a platform to help improve existing authentication methods.

**KEYWORDS**

Analytical methods; authentication; Halal; Kosher; meat; meat products

## Introduction

Meat and meat products are an important part of human diet since they contain proteins and essential amino acids as well as minerals, vitamins, and micronutrients which are very essential for the development, growth, and good activities of the body organs (FAO 2014; Pimentel and Pimentel 2003). Especially, high nutrient containing foods such as meat and meat products are of special benefit for children and women as well as for the communities infected with HIV or AIDS patients (FAO 2014). Consequently, many countries consider the meat-based diet as one of the basic and significant nutritious food that comprises an important part of the food manufacturing industry. Thus, approximately 2 billion people depend primarily on meat-based food worldwide (Pimentel and Pimentel 2003). The selection and consumption of meat depend on several factors such as religious faiths, age groups, geographical zones and consumers' purchasing ability. Among these, religion is the most vital aspect to choose meat particularly for the Muslims and the Jews communities. For instance, the Muslims prefer Halal branded meat and meat products while the Jews prefer Kosher ones (El Sheikha et al. 2017).

The demand of Halal and Kosher branded food products has become a new trend not only due to the religious belief but also for their assured quality. Protecting the sanctity of Halal and Kosher branded food products, especially meat and meat products, has become a global issue because of rapid expansion of Halal and Kosher food markets in all corners of the world (Ali et al. 2015c; Rahman et al. 2014). Currently, the Muslim population has reached to 1.9 billion (WPR 2019) and the revenue from global Halal food business is forecasted to increase from about 1.25 trillion US$in 2016 to about 2.57 trillion US$in 2024 (Shahbandeh 2019). On the other hand, the global Jewish population was estimated about 14.41 million and world Jewry increased at about half the general population growth rate (DellaPergola 2017). Thus, consumer trend to Halal and Kosher products is increasing day by day. Most people who consume Kosher products believe that the Kosher label refers to higher-quality food. Now a days, many non-Jewish consumers buy Kosher food because they consider it to be of better quality

CONTACT M. A. Motalib Hossain motalib_123@yahoo.com Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, Kuala Lumpur 50603, Malaysia.

and healthier (Sullivan 1993). Therefore, customers are ready to pay higher price for Halal and Kosher foods because of its special requirements in manufacturing and supply chain which have made the products susceptible to adulteration (Ali et al. 2015c). There are specific guidelines to follow about food preparation and diet in the holy books of both Islam and Judaism and the followers also obey many restrictions and rituals regarding cleanliness of foods (Eliasi and Dwyer 2002). Thus, proper labeling of constituents in meat and meat products and their subsequent field monitoring are considered as seriously important issues in modern days to prevent food forgery, safeguard consumers trust and maintain sustainable food businesses. Considering the need, most of the countries have regulatory bodies for tracing and tracking of adulterants such as lower grade or lower priced meats mixed with the higher priced ones (Al Amin 2015). Consequently, numerous authentication techniques for detecting species in the meat products have been reported to fulfill the demand.

This review focuses on the discussion of available reliable techniques to authenticate meat and meat products with brief discussion about Halal and Kosher laws with a view to creating consumer awareness and protect them from being deceived. The objective of this article is to elucidate the present situation, day by day progress and future challenges of Halal and Kosher meat and meat products authentication schemes.

## What is Halal and Kosher?

The food permitted to consume for the Muslim is defined as Halal food. The word “Halal” is an Arabic word that is referred to as what is permitted and lawful by the Shariah (Islamic Law) (Mohamed et al. 2016). However, any food which Muslim is allowed to consume as per the description of the Quran and Hadith (the saying and practice of Prophet Muhammad (peace be upon him)) and the fiqah (interpreted by Muslim scholars) is known as Halal (Regenstein, Chaudry, and Regenstein 2003). The Muslim believes that Islamic Shariah allowed only the food which are beneficial for health and restricted that are harmful for the body as described in the holy book Quran (Anonym 2017; Halalce 2017).

> *“O mankind! Eat of that which is lawful and good on the earth, and follow not the footsteps of Shaitan (Satan). Verily, he is to you an open enemy”* (*Quran 2.168*).

> *“O you who believe (in the Oneness of Allah Islamic Monotheism)! Eat of the lawful things that We have provided you with, and be grateful to Allah, if it is indeed, He Whom you worship”* (*Quran 2.172*).

> *“He has forbidden you what dies of itself (carrion), and blood, and the flesh of swine and that over which any name other than (that of) Allah has been invoked”* (*Quran 2. 173*).

Haram (non-Halal) is the opposite of Halal, which means prohibited or unlawful (Majid et al. 2015). In fact, the terms Halal and Haram are, universal that are applicable in all aspects of life. Thus, Halal animals include cows, buffaloes, goats, sheep, camel, duck, goose, chicken, ostrich, turkey etc. if they are slaughtered according to Sharia law. To be determined as Halal or Haram it is important to know how the food is obtained and processed while other factors may also be considered. Any food that directly causes a bad effect or has the potential to do so on the mind, body or spirit is “haram”: “unauthorized or illicit.” The list of various Haram or prohibited foods include pork and its by-products, meat of animals that are shot or died of disease and those which were not slaughtered or slaughtered in a name other than that of Allah, products prepared with alcohol, blood and other intoxicating substances (Eliasi and Dwyer 2002). Other non-Halal foods include horse, dog, cat, rat, donkey, frog, carnivorous animals etc. Despite being Halal, some foods may also be unsafe for human consumption. Therefore, food and food products that do not play a role in preserving human’s life and health are also considered haram or illicit. Similarly, lard oil (pork fat) is being used in food and cosmetics as safe substances but it fails to fulfill religious sentiments. Therefore, being Muslim one should always look for Halal products to meet the religious requirements that would preserve bodies and souls (Majid et al. 2015; Muhammad, Isa, and Kifli 2009). Islamic law emphasizes on cleanliness throughout the holy Quran; ‘eating only Halal foods will keep the faithful clean and pure’ (Eliasi and Dwyer 2002).

The Hebrew word ’Kosher’ may be similar in meaning of English word ’fit’ in the sense of proper or suitable. But we should know that the fitness is mostly ceremonial (Masoudi 1993; Sullivan 1993). The word "Kosher" which usually refers to food that accomplishes the standards of kashrut, is also often used to indicate ritual objects that are prepared according to Jewish law and can be used for ritual purposes. The Kosher dietary laws originally come from biblical law whereas, "Kashrut" comes from the Hebrew root Kaf-Shin-Reish, meaning fit, proper or correct (Rich 1995; Sullivan 1993). Kashrut is the body of Jewish religious laws concerning the suitability of food and how those foods must be prepared and eaten etc. It also describes the manner of slaughtering of allowed animals. Food is Kosher, or allowed to eat, when prepared in compliance with the conditions set by Judaic law. Food unsuitable to eat is designated as ‘non-Kosher’ or ‘treif’ which means unclean (Eliasi and Dwyer 2002).

An animal’s flesh can only be declared as Kosher provided that the animal is a ruminant (one that chews its cud) and have split hooves. For example, pigs having cloven hooves are non-Kosher as they are not ruminants. Thus, cow, goat, deer, bison, and sheep are permitted for Jews. Poultry such as chickens, turkeys, ducks, and geese do not have front toes, they possess a craw with a double-lined stomach and are considered potential Kosher depending on the manner of slaughtering. Birds of prey, non-domesticated birds, and insects are not allowed to eat. Moreover, the animal is examined whether there is any blemish suggestive of its illness, making the animal non-Kosher. Animals which died in a natural process or which were slaughtered in a way other than the defined one are also considered non-

Kosher (Eliasi and Dwyer 2002). It is prohibited to consume both the meat and the milk or dairy together of such sources (Rich 1995).

In Islamic law, Dhabiha is the prescribed method of ritual slaughter of all lawful Halal animals. Dhabiha meat refers to the meat that is slaughtered in the shariah manner and the name of Allah must be announced upon slaughter of each animal separately. In Shechita, a blessing to God is pronounced before starting a long period of slaughtering and if there is not a long interruption, it is thought that this blessing covers all the animals slaughtered in that period. There is a standard form for blessing which is followed before most Jewish rituals. In Judaism it is usually followed that for rituals having an associated blessing, if one fails to recite the blessing, the ritual is still valid; similarly, even if the shochet omits the blessing before Shechita, the slaughter is considered valid and the meat is Kosher. If the animal is slaughtered and prepared according to Halal rules, all organs or parts of the carcass may be eaten without any restrictions. Muslims are allowed to consume all the entire animal from a Halal-slaughtered and -dressed animal except blood. On the other hand, Kashrut does not allow eating the chelev (certain types of fat) and gid hanosheh (the sciatic nerve), and therefore it is obligatory for the Jews to follow a process called nikkur (or, in Yiddish, traibering) for the hindquarters of a Kosher animal if they want to consume it. However, nikkur is not regularly practiced outside of Israel because it is expensive and requires time, therefore in other parts of the world, they usually sell the hindquarters of Kosher-slaughtered animals in the non-Kosher market (Rovinsky and Cohen 2018). To the best of our knowledge, until now, no method has been developed to verify whether the meat comes from slaughtered or non-slaughter animals.

### *Food safety and traceability*

The increasing number of food fraud incidences has been the major concerns for the food safety and environmental forensic professionals around the globe. Recently, 'food safety' has been defined by World Health Organization (WHO) as a term that in general refers to means and approaches to ensure that the production, preservation, distribution and consumption of food are performed in a safe and harmless manner. However, any single definition of food safety might be oversimplified since, viewpoints and descriptions of safe food may differ and be diversified depending on consumers, academicians, industry and special interest groups (El Sheikha 2015). From consumers point of view, the concept of 'safe food' is generally quite simple and practical. For instance, some may define safe food as food that does not cause any disease while to some others safe food should be free from any contamination and/or adulteration (Elmi 2004). Halal and Kosher food products are a new and better option for those demanding for safer, cleaner and healthier foods.

However, since food safety is an integral part of food quality, in recent times, tracking and tracing systems have emerged as the most important tools used to ensure food safety. With the use of a reliable traceability system, regulatory authorities or food companies are able to track and trace any foodstuff that does not meet consumer expectations or is not compliance with the related applicable regulations of the country. The main objective of a traceability system is to disclose a product's story, i.e., specify a definite product batch and the ingredients used in its production and follow the same throughout the production and distribution chain. With the increase in global population and ever developing consumer awareness toward food quality, food safety, and authenticity, food production, marketing and distribution as well as food integrity issues have never been more important in human history. Over the decades since the introduction of molecular biology and biotechnology, remarkable improvements have been made to enhance food production, enrich food nutrition, improve food quality and ensure food authenticity in global perspectives (El Sheikha, Levin, and Xu 2018).

## The importance of Halal and Kosher authenticity in meat and meat products

Both Halal and Kosher meat products' business is expanding day by day worldwide due to consumers' new trend of food habit. Only the Muslim communities do not consider Halal foods as their sole religious requirements, the non-Muslims now also started choosing this type of food as they perceive that Halal foods are much cleaner, congenial to health and safe (Zulfakar, Anuar, and Talib 2014). The Global market for certified Halal food products including meat and meat products is rapidly increasing and it has a huge potential for expansion as the Halal products are receiving worldwide recognition as a scale for food safety and quality assurance (Majid et al. 2015). The projected growth of Halal product market is expected to increase to over 58.3 billion U.S. dollars by 2022 from 45.3 billion in 2016 (Shahbandeh 2019). A large number of consumers prefer food products compliant with Islamic law containing meat from religiously slaughtered animals. Halal food products are a new and better option for those demanding for safer, cleaner, and healthier foods (Zulfakar, Anuar, and Talib 2014).

Similarly, Kosher is another growing food trend worldwide dealing mainly with quality. Over the past decade, the demand of Kosher (food permitted to the Jews) food products has been increased drastically introducing a new trend among the modern people (Solanki 2016). Kosher market is expanding particularly in the United States and Europe (Jayalal 2015; Lever et al. 2010). In a recent study it was estimated that among the total Kosher food consumers (12.5 million) in the USA, only 20% are Jewish while the rest 80% are non-Jewish consumers (Yang 2017). It is estimated that approximately 40% of the shelf's products in the supermarkets of the USA are Kosher and 125,000 Kosher products are available in US supermarkets and about 3,000 more are being included each year and the number of Kosher consumers is more than 45 million worldwide (Buckenhüskes 2015; KLBD 2017). It has been reported that over 10,000 American companies manufacture Kosher products, Europe

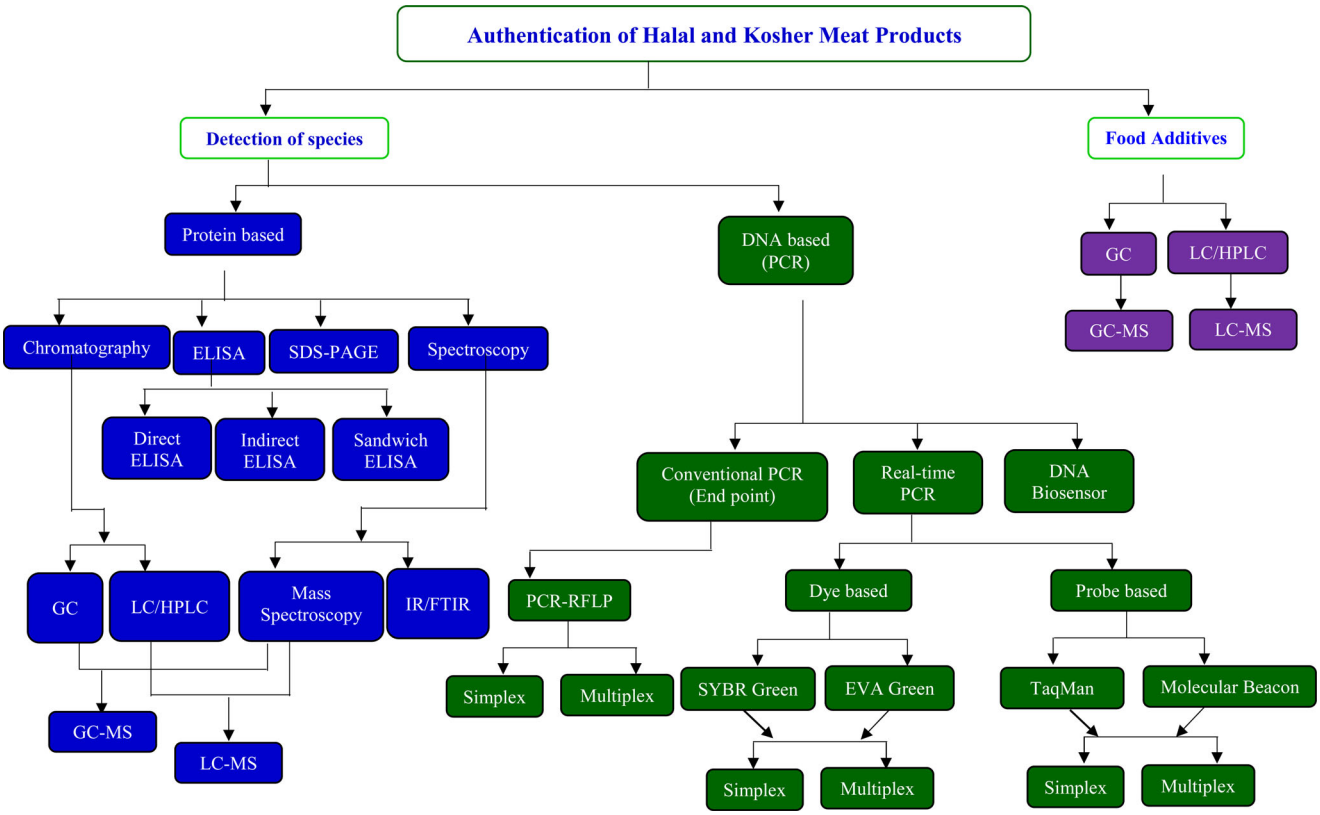


**Figure 1.** A summary of Halal and Kosher meat and meat products authentication techniques.

being the second highest contributor in world Kosher food market (Solanki 2016). According to annual food sales report, Kosher products comprised of about 200 billion US dollars (40%) among the total food sales of 500 billion US dollars (Buckenhüskes 2015). Mintel conducted a consumer survey among adults who usually purchase Kosher food. They pointed out that there are three main reasons for which people buy Kosher food. First, food quality (62%), the second is 'general healthfulness' (51%) and the third is food safety (34%) (Mintel 2009).

Why are consumers concerned about the authenticity of Halal and Kosher products globally? The manufacturing of Halal and Kosher meat and meat products involves many crucial steps from farm-to-fork to ensure that there is no chance of cross contamination as well as adulteration with non-Halal and/or non-Kosher ingredients throughout all production steps. However, the food manufacturer is often not aware of the demand of the Muslim and Jewish consumers or often conduce intentional adulteration for economic benefits (Nakyinsige et al. 2012). Thus, a comprehensive and perfectly managed supply chain system must be ensured to make Halal and Kosher meat products easily available and for this purpose, an authentic analytical tool is crucially needed.

## Advancement in the analytical methods to detect species in meat and meat products

Halal and Kosher authenticity issues are closely related with the development of innovative analytical methods that are the most important tool for regulators to make confirmatory scientific decisions. Although various analytical techniques useful in the speciation and authentication of food products have been illustrated in different articles, our discussion will focus on recent development on the analytical methods both in the field of chemical and biological assays. Several species detection techniques have been developed so far including immunochemical (Tukiran et al. 2016b; Venien and Levieux 2005), spectroscopic (Kurniawati, Rohman, and Triyana 2014; Boyacı et al. 2014), chromatographic (Eerola et al. 2007), electrophoretic (Naveena et al. 2017), gas chromatography coupled with mass spectrometry (GC/MS) (Nurjuliana et al. 2011), liquid chromatography coupled with mass spectrometry (LC/MS) (von Bargen et al. 2013), and lateral flow assay (LFA) (Hsieh and Gajewski 2016; Magiati et al. 2019) techniques. DNA-based methods are also available such as conventional Polymerase Chain Reaction (PCR) (Ahamad et al. 2019; Sultana et al. 2018b), PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) (Erwanto et al. 2014; Sultana et al. 2018a) real-time PCR (Iwobi et al. 2015; Linacero et al. 2020), DNA barcoding (Ahmed et al. 2018; Hajibabaei et al. 2006) and DNA biosensors (Zhang, Wang, and Jiang 2011). An overview of the major species identification methods is depicted in Figure 1 and their comparative features are summarized in Table 1.

## Protein based methods

The protein-based methods mainly involve immunological assays. These methods have been widely used to verify the species origins of animal materials in meat and meat products (Ali et al. 2018). To be applied for regulatory purposes,

**Table 1.** Existing Halal and Kosher meat and meat products authentication techniques and their key features.

| Technique name | Major features | Advantages | Limitations | Detection limits | Target species | References |
|---|---|---|---|---|---|---|
| **Protein based methods** | | | | | | |
| Chromatography | Detection is performed based on differential adsorption between the mobile and stationary phases. It produces unique chemical fingerprints that differentiate and authenticate foods. The authentication is based, on identification of minimal analytical differences between patterns or identification of unique marker compounds. | • Rapid and reliable<br>• Able to separate chemically similar compounds in complex food matrices<br>• Very pure compounds can be recovered<br>• Heat labile or delicate products can be separated | • Low throughput<br>• Creates problem due to irreversible adsorption<br>• Should be handled with care as it's parts are costly and sensitive<br>• High operating pressure may be required to get complete separation | 0.50%<br>0.55%<br>0.13%<br>0.24% | Animal protein<br>Pork and horse meat<br>Pork<br>Pork and horse meat | Aristoy and Toldrá (2004)<br>von Bargen et al. (2013)<br><br>von Bargen, Brockmeyer, and Humpf (2014) |
| ELISA | ELISA is an immunological technique that involves an enzyme (a protein that catalyzes a biochemical reaction) to detect the presence of an antibody or an antigen in a sample. | • Simple, fast and easy to handle.<br>• Sensitive and low cost<br>• Suitable for both qualitative and quantitative measurement. | • A positive result may occur if an unrelated antibody reacts with the antigen nonspecifically<br>• Multiplexing not possible<br>• Not suitable for severely processed samples due to degradation of protein based biomarkers<br>• Enzyme/substrate reaction is short term so microwells must be read as soon as possible<br>• Negative controls may indicate positive results if blocking solution is ineffective | 126 and 250 ppm<br>0.01- 0.05%<br>0.78 ng/mL<br>0.05 μg/mL | Chicken or turkey and pork<br>Pork<br>Pork<br>Pork | Berger et al. (1988)<br>Liu et al. (2006)<br>Doi et al. (2009)<br>Tukiran et al. (2016a) |
| SDS-PAGE | Separates proteins and peptides based on their molecular weight | • Simple and easy<br>• Inexpensive<br>• Require small amount of samples<br>• Sensitive and can separate 2% difference in mass | • Need to prepare reference samples<br>• Peptide biomarkers degrade easily<br>• Unable to detect closely related species due to structural similarities<br>• Non-quantitative | Not given<br>Not given<br>Not given | Marine fish<br>Cattle, sheep, lambs, goats, red deer and rabbits<br>Moray eel meats | Martinez and Jakobsen Friis (2004)<br>Montowska and Pospiech 2007<br>Huang et al. 2010 |
| Spectroscopy | Detection is performed based on measurement of vibrational energy of atoms or groups of the analysts generated by the absorption of specific IR wavelengths (Rodriguez-Saona and Allendorf, 2011). | • Require little amount of sample<br>• Analysis is rapid<br>• Eliminate the use of hazardous solvents | • Highly pure samples are required<br>• Unable to detect species under complex matrices<br>• Limited sensitivity | 0.94 – 1%<br>Not given<br>Not given | Beef<br>Pork<br>Beef and horse meat | Zhao, Downey, and O'Donnell (2014)<br>Kurniawati, Rohman, and Triyana (2014)<br>Boyacı et al. (2014) |
| **DNA based methods** | | | | | | |
| Conventional PCR (End point) | Target amplicons are amplified using extracted target DNA and a pair of species specific primers followed by identification using electrophoretic | • Highly stable biomarkers<br>• Both simplex and multiplex are possible | • Non-quantitative<br>• laborious and time consuming because post amplification analysis is required<br>• Poor Precision | 1.0 pg<br>0.01–0.02 ng (pure state) and 1% (admixture)<br>0.01% (pure state) | Chicken, beef, pork and mutton<br>Cat, dog, pig, monkey and rat meats<br>Horse, sheep, poultry, pork cow and soybean | Zhang (2013)<br>Ali et al. (2015c)<br>Safdar and Junejo (2016) |

(*continued*)

**Table 1.** Continued.

| Technique name | Major features | Advantages | Limitations | Detection limits | Target species | References |
|---|---|---|---|---|---|---|
| | separation such as agarose gel or capillary electrophoresis chips. | • Highly sensitive, specific, robust, and reproducible<br>• Relatively cheap<br>• Amplified products could be authenticated using sequencing and RFLP analysis | • Low resolution<br>• Size-based discrimination only | 0.02 ng (pure state) and 0.1% (admixture) | Beef, buffalo and pork | Hossain et al. (2017a) |
| PCR-RFLP | Targeted DNA sites are amplified by conventional PCR assay. The PCR products are digested with specific restriction enzyme(s), generating different size-length DNA fragments which are separated and visualized by gel electroporesis. | • Very simple and easy to perform.<br>• No need of advanced lab equipment<br>• No need of prior sequence information<br>• Inexpensive | • Large amount of samples is required.<br>• Time consuming and laborious<br>• Specific endonuclease is required<br>• Difficult in the detection of accurate variation where various single nucleotide polymorphisms (SNPs) affect the same restriction enzyme recognition site. | 0.0001ng (pure state) and 0.01% (admixture)<br>Not given<br>Not given<br>0.1% (admixture) | Pork<br>Cow, chicken, turkey, sheep, pig, buffalo, camel and donkey<br>Beef, carabeef, chevon, mutton and pork<br>Beef, buffalo and pork | Ali et al. (2012a)<br>Haider, Nabulsi, and Al-Safadi (2012)<br>Kumar et al. (2014)<br>Hossain et al. (2016) |
| Real-time PCR | Amplification progress of target DNA fragments are monitored during PCR reaction by recording the amount of fluorescence emission from fluorescent reporter at each cycle. | • Highly stable biomarkers<br>• Both simplex and multiplex are possible<br>• Both qualitative and quantitative<br>• Highly specific and sensitive<br>• Robust, efficient and reproducible<br>• No post-PCR analysis is required | • Costly and trained person is required to handle<br>• Design of probe, selection of fluorescence and optimization are complicated | 0.01% (admixture)<br>0.15 ng<br>20 genome equivalents<br>0.003 ng (pure state) and 0.1% (admixture)<br>0.5%<br>$2.9 \times 10^{-13}$ mol/L | Pork<br>Duck, pig and chicken<br>Beef and pork<br>Beef, buffalo and pork<br>Pork<br>Not given | Ali et al. (2012)<br>Cheng et al. (2014)<br>Iwobi et al. (2015)<br>Hossain et al. (2017b)<br>Ali et al. (2011)<br>Zhang, Wang, and Jiang (2011) |
| Biosensors | Short-length single-stranded DNA probe is immobilized on to the surface of the signal transducer. Specific target is hybridized with the probe DNA resulting changes in optical or electrochemical properties which can be measured by the signal intensity. | • Portable<br>• Rapid<br>• Very simple and easy to handle<br>• Low-cost<br>• Highly sensitive | • Pure DNA sample is required<br>• Most cannot achieve multiple detection | 0.5%<br>~20.33 ng/μL, ~78.68 pg/μL and ~23.63 pg/μL<br>0.58 μg/mL | Pig<br>Pig, chicken, and cow<br>Pig | Ali et al. (2014a)<br>Ahmed et al. (2010)<br>Hartati et al. (2019) |

**Table 2.** Detection of species in Halal and Kosher meat and meat products using protein based methods.

| Methods | Target species | Target protein | Type of antibody | Limit of detection | References |
|---|---|---|---|---|---|
| Direct ELISA | Chicken and pork | Skeletal muscle tissue heating to 120 degrees C for 15 min | Polyclonal | 126 and 250 ppm | Berger et al. (1988) |
| Indirect competitive ELISA | Poultry, rat, kangaroo and horse | Muscular tissue and heated to 100 or 120 °C for 30 min | Polyclonal | 1-5% | Renčová, Svoboda, and Necidova (2000) |
| Sandwich ELISA | Pig | Sarcoplasmic extracts | Monoclonal | 1% | Martin et al. (1988) |
| Sandwich ELISA | Ovine | Muscle proteins to cooked ($100^0$ C, 30 min) | Monoclonal | 0.25-0.5% | Liu (2006) |
| Sandwich ELISA | pork | Albumin | Not given | 1% | Martin, Chan, and Chiu (1998) |
| Indirect ELISA | Pork | Muscle protein | Monoclonal | 0.5% | Chen and Hsieh (2000) |
| Sandwich ELISA | Pork | Skeletal muscle 132 °C for 2 h | Monoclonal | 0.05-0.1% | Liu et al. (2006) |
| Commercial ELISA kit | Pork and beef | – | – | 10.0% (pork) and 1.00% (beef) | Perestam et al. (2017) |
| Commercial ELISA kit | Beef, poultry, horse and deer | – | Monoclonal | – | Ayaz, Ayaz, and Erol (2006) |
| dot-ELISA | Bovine, chicken, swine and horse | Albumin | – | 0.6% | Macedo-Silva et al. (2000) |

species authentication techniques, especially enzyme-linked immunosorbent assays (ELISAs), have been used widely as it is specific, simple, sensitive, and offers high throughput screening with reduced cost and time (Carrera et al. 2014). It is well suited technique for larger number of samples which can be handled at a time (Singh et al. 2014). According to ELISA principle, either antibody (Ab) or antigen (Ag) is fixed to a surface followed by measurement of antigen-antibody interactions by the assistance of the labeled enzyme (E) which converts specific substrate into a colored product. The measurement of the produced color is an indicator for the identification and quantification of the sample (Pokhrel 2015). The most commonly used ELISA techniques for the authentication of meat and meat products include indirect and sandwich ELISA (Asensio et al. 2008). The different types of ELISA methods are described below:

## *ELISA assays*

ELISA assay can be used in both qualitative and quantitative analysis. In qualitative analysis, assays provide either positive or negative result for the sample, but in the quantitative assay, a standard curve is generated by interpolating the fluorescence or optical density of the serially diluted antigen concentration (Asensio et al. 2008). The development of antibodies against the target antigens is mandatory for the ELISA analysis to produce rapid results. Two types of antibodies are used in the ELISA technique to authenticate food ingredients, namely monoclonal (Chen, Hsieh, and Bridgman 2008; Hsieh and Bridgman 2004; Liu et al. 2006) and polyclonal (Berger et al. 1988; Hsu, Pestka, and Smith 1999; Smith et al. 1996) antibody. Polyclonal antibodies (PAbs) are more suitable for the analysis of denatured protein samples as they are able to recognize the antigens from a mixture of different epitopes with little changes in the property of antigen, such as denaturation or polymerization (Asensio et al. 2008). However, PAbs have some limitations including limited yield, variable affinity and extensive purification step needed to overcome cross-reactivity for the detection of specific-species. On the contrary, monoclonal antibodies (MAbs) are produced homogenously by using hybridoma techniques with high yield, specific biological activity and high specificity (Asensio et al. 2008).

Until now, various ELISA reports have been documented for the authentication of food using both MAbs and PAbs based on structural and soluble proteins of the muscle cell (see Table 2). Some factors like high temperature may denature the protein and thus they cannot be detected in immunoassays and therefore the identification of animal species on processed food (by heat treatments etc.) becomes difficult. To overcome this limitation, Berger et al. (1988) raised PAbs against the antigen of chicken and pork muscle tissues, which are heat resistant. They found that isolated antigens were immunoreactive under 120° C for 15 min and sensitivity of chicken and pork ELISA were 126 and 250 ppm level, respectively. Similarly, Renčová, Svoboda, and Necidova (2000) also developed ELISA method for the identification of heat-treated samples. Poultry, rat, kangaroo and horse species were successfully identified with a sensitivity of 1-5% by developing PAbs against muscular tissue which was heated at 100° or 120° C for 30 min. To detect the adulterated pork in beef mixture, sandwich ELISA technique was introduced by raising PAbs against muscle soluble protein with the detection limit of 1% adulteration level (Martín et al. 1988). Later, Liu (2006) developed the sandwich ELISA using MAbs raising from soluble myofibril proteins extracted from heat-treated ovine muscle. The ELISA system was highly reactive to heat-treated (100° C for 30 min) sheep muscle proteins. The detection limits were 0.25%, 0.5% and 0.5% (w/w) for cooked ovine muscle adulterated with chicken, beef, and pork, respectively. ELISA technique was also applied for the authentication of certain food products like frankfurter, cooked salami and fermented sausage (Ayaz, Ayaz, and Erol 2006) and hamburger (Macedo-Silva et al. 2000).

Researchers also developed ELISA methods for the quantitative evaluation of adulterated meat samples. For examples, ELISA method was approached for the quantitative

measurement of raw pork in the admixture of raw beef with the quantification limit up to 1% (Martin, Chan, and Chiu 1998). Chen and Hsieh (2000) reported ELISA technique for the quantification of pork in various heat-treated meat products such as sausage bologna ham, salami spread franks and luncheon meat using MAbs which was raised against heat-stable muscle protein of pig. The limit of detection was found 0.5% (w/w) porcine material in various meat mixture and the accuracy of the developed method was confirmed by comparative study with commercial PAbs test kit (Chen and Hsieh 2000). Similarly, Liu et al. (2006) developed MAbs based quantitative Sandwich ELISA assay for the evaluation of porcine material in raw and thermal-treated (132° C for 2 h) meat samples with the lower detection limit of 0.05% (w/w) of pork in adulterated chicken mixture and 0.1% (w/w) porcine in beef admixture. Currently, the ELISA test kits of specific meat species are commercially available for the reliable analysis of raw, processed, cooked meat, meat product, and feedstuff (Asensio et al. 2008; Heo et al. 2014).

Perestam et al. (2017) conducted a comparative study between ELISA and DNA-based method (real-time PCR) based on specificity, sensitivity, time, cost and purpose of use. They found that both methods are suitable for detecting the species origin in raw meat and meat products, but ELISA is not suitable for the identification of species in highly processed food particularly when a lower detection limit is a requisite. Other researchers also commented on lower sensitivity of the ELISA assay and suggested that it is not suitable for the differentiation of species in mixed matrices particularly in closely related species (Martin, Chan, and Chiu 1998; Martín et al. 1988). Moreover, immunoassays were often interrupted due to cross-reactions occurrence between closely related species, since these techniques are based on the raised antibodies against a specific protein (Di Pinto et al. 2005; Fajardo et al. 2010).

Although ELISA method offers enormous advantages, the technique also suffers from some limitations. For example, heat or other processing treatments might denature the target Ag resulting in distortion of epitopes' original forms that interferes with the binding of Ab to the specific antigen. Ag and Ab binding may also be interrupted especially in the severely processed foods, since the food ingredients such as carbohydrates, lipids, nucleic acids, salts, and other components might exert the inhibitory effects. In addition, the quantity of Ag in the food products is also important. The amount below the LOD level may make the method unable to detect it (Nhari et al. 2019; Mohamad et al. 2016).

## DNA based techniques

Now a days, application of DNA based detection methods gained much popularity for the authentication of Halal and Kosher foods, as it is highly sensitive and specific compared to immune assays (Aida et al. 2005; Erwanto et al. 2014). DNA based techniques are more advantageous due to greater biomarker stability and extraordinary sensitivity; the biomarker targets are significantly amplified from a single copy or a few copies into quantities that could be easily detected (Bansal et al. 2017; Kumar et al. 2015; Mohamad et al. 2018). The sensitivity of this method is further strengthened as there is a uniform information content; several nucleic acids such as mitochondrial DNA (mtDNA), are present everywhere in multiple copies, and polymorphic features are more widely available throughout the genome. These methods can detect the species even in complex and severely processed food matrices where DNA is broken down making it inadequate for identification. To detect species DNA in extremely processed food, PCR techniques have been improved by targeting shorter length DNA fragments ($\geq$150 bp) which are very stable under extreme food processing treatments (Ali et al. 2016; Ali et al. 2015b).

The specific gene is selected to design the species-specific primer set for a successful PCR assay. The selection of gene depends on the inter species conserved region and intra species hyper variable region which facilitate to increase the specificity of the PCR assay. Generally, mitochondrial Cytb gene is targeted to design the PCR biomarkers. This gene contains both conserved and variable regions which facilitate to clarify deeper evolutionary relationship and resolve divergence at population level, respectively. Besides this, researchers also use other mitochondrial-encoded genes namely, 12S rRNA, 16S rRNA, D-loop, NADH dehydrogenase subunit 5 (ND5), and TPase6/ATPase8 depending on the target species as well as assay design (Mohamad et al. 2013).

Several PCR techniques have been developed and validated for the discriminatory detection of different species in raw as well as normal and extremely processed food matrices. Two main types of PCR methods are being used for food authentication, namely, end point (conventional) and real-time PCR. Both methods can adapt simplex and multiplex systems. Simplex system refers to single species detection technique whereas multiplex system offers multiple species detection in a single assay platform.

### *End point PCR*

In End point (EP) PCR, usually a target region of a mitochondrial gene is amplified using a set of species-specific primer pairs along with a buffer, magnesium chloride and specific enzymes and then the amplified product is visualized on an agarose gel using ethidium bromide or other non-carcinogenic dyes (Ali et al. 2018). EP-PCR gives results only at the end of reaction in the form of bands in the gel. This is a simple, reliable, sensitive and low-cost method, and has been widely used to authenticate animal species from various sources. The various steps in the development of EP-PCR are presented in Figure 2. Several simplex and multiplex EP-PCR methods have been reported for species authentication (see Table 3). For example, Barakat, El-Garhy, and Moustafa (2014) introduced porcine specific simplex PCR method targeting two different mitochondrial genes; cytb (117 bp) and D-loop (185 bp), for raw and cooked sausage samples using automated QIAxcel capillary electrophoresis system. The developed method was highly sensitive, hence up to 0.01% pork adulteration in beef could

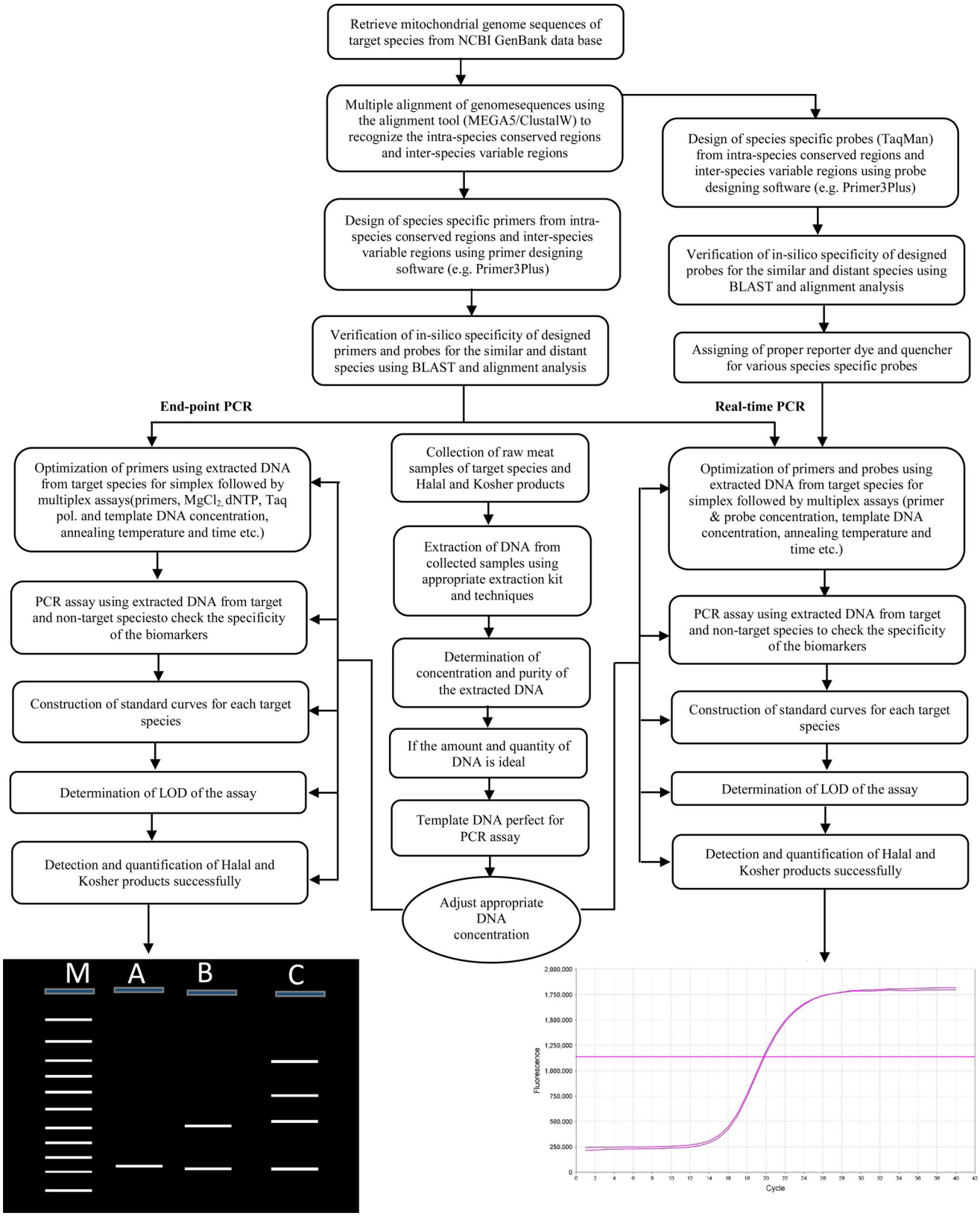


**Figure 2.** Various steps involved in the development of end-point and real-time PCR (qPCR) systems to authenticate Halal and Kosher meat and meat products.

be detected by using species-specific genes under raw and heat-treated samples. Another powerful, simple and sensitive PCR-RFLP method was reported for the detection of porcine material in meatball. In this assay, cytb gene targeted 359 bp amplified product was digested with *BseD*I restriction enzymes which generated 131 and 228 bp fragments. A survey on Indonesian beef meatball products found porcine positive in nine samples out of twenty (Erwanto et al. 2014). Kumar et al. (2014) used a pair of forward and reverse primers from the conserved region of mitochondrial cytb gene

**Table 3.** Detection of species in Halal and Kosher meat and meat products using DNA based methods.

| Methods | Target species | Target gene | State | Target size (bp) | Limit of detection | References |
|---|---|---|---|---|---|---|
| PCR-RFLP | Pork | Cyt b | Normal | 360 | Not given | Aida et al. (2005) |
| Simplex PCR | Pork | 12S rRNA | Normal | 387 | Not given | Che Man et al. (2007) |
| Simplex PCR | Cat, dog and rat or mouse | 12S rRNA | 133 °C for 20 min at 300 kPa | 108, 101, & 96 | 0.1% | Martin et al. (2007) |
| Simplex PCR | Pork | Cytb & D-loop | Cooked | 117 & 185 | 0.01% | Barakat, El-Garhy, and Moustafa (2014) |
| PCR-RFLP | Pork | Cytb | Normal | 359 | 0.1% | Erwanto et al. (2014) |
| PCR-RFLP | Pig, cattle, goat, buffalo, and sheep | Cytb | Normal | 609 | Not given | Kumar et al. (2014) |
| PCR-RFLP | pork, goat, beef, buffalo, chicken, rabbit and quail | Cytb | Normal | 359 | 1-5% | Murugaiah et al. (2009) |
| PCR-RFLP | Pork | D-loop | Normal | 276 | 1% | Mane, Mendiratta, and Tiwari (2013) |
| Simplex PCR | Dog | Cytb | Autoclaved for 2.5 h | 100 | 0.2% | Rahman et al. (2014) |
| PCR-RFLP | Cat | Cytb | Autoclaved for 2.5 h | 69 | 0.01% | Ali et al. (2015) |
| PCR-RFLP | Macaque | D-loop | Normal | 120 | 0.1% | Rashid et al. (2015) |
| PCR-RFLP | Dog, cat, horse and donkey | Cytb | Normal | 672, 808, 221 and 359 | – | Abdel-Rahman et al. (2009) |
| Multiplex PCR | Pig, cattle, chicken and horse | Cytb | Normal | 412, 292, 239 & 451 | 0.1 ng | Bai et al. 2009 |
| Multiplex PCR | Chicken, goat, cow, camel and donkey | Cytb, 12S rRNA, ND 2 | Normal | 183, 157, 274, 200 & 145 | 0.1 ng | Nejad et al. (2014) |
| Multiplex PCR | Chicken, beef, mutton and pork | Cytb | Cooked & further-processed foods | 216, 263, 322 & 387 | 1 pg | Zhang (2013) |
| Multiplex PCR | Pork, lamb/mutton, chicken, ostrich, horsemeat and beef | cyt b, cytochrome oxidase I (COI), & 12s rRNA | Highly processed food | 100, 119, 133, 155, 253, & 311 | 7 fg | Kitpipit, Sittichan, and Thanakiatkrai (2014) |
| Multiplex PCR | Pig, cattle, poultry (chicken and turkey), equine (horse and donkey) | Cytb, 12S rRNA | Processed food | 212, 256, 183 & 439 | Not given | Ilhak and Guran (2015) |
| Multiplex PCR | Cat, dog, pig, monkey and rat | ND5, ATPase 6, & cytb | $121^0$ C for 2.5 h | 172, 163, 141, 129 & 108 | 0.01–0.02 ng (pure) & 1% (admixed) | Ali et al. (2015) |
| Multiplex PCR | Horse, soybean, sheep, poultry, pork and cow | cyt b, lectin, 12S rRNA, 12S rRNA, ATPase 6 & ATPase 8 | Normal | 85, 100 119 183 212 & 271 | 0.01% | Safdar and Junejo (2016) |
| PCR-RFLP | Beef, buffalo and pork | Cytb and ND5 | Autoclaved for 2.5 h | 73, 90, 106, 120, 138 and 146 | 0.1% | Hossain et al. (2016) |
| Multiplex PCR | Cow, buffalo, chicken, cat, dog, pig and fish | Cytb, ND5 and 16s rRNA | Boiling, autoclaving and microwave cooking | 73, 85, 103, 120, 138, 161 and 198 | 0.01 – 0.001 ng and 0.5% | Hossain et al. (2019) |
| Multiplex PCR | Crocodile | Cytb and ATP6 | Boiling, autoclaving and microwave cooking | 77 and 127 | 0.01 – 0.001 ng and 1% | Ahmad Nizar et al. (2018) |
| Simplex qPCR (TaqMan) | Pork | 12S rRNA | Normal | 411 | 0.5% | Rodríguez et al. (2005) |
| Simplex qPCR (TaqMan) | Pork, beef, mutton, chicken and horse | Cytb | Normal | Not given | 100 fg | Tanabe et al. (2007) |
| Multiplex qPCR (TaqMan) | Chicken, pork, turkey, horse, beef, sheep (mutton) and goat | Cytb, Beta-actin-gen & Prolactin receptor | Normal | 76, 80, 83, 85, 96, 101 & 140 | 2% | Köppel, Zimmerli, and Breitenmoser (2009) |
| Simplex qPCR (TaqMan) | Pork | Cytb | Cooked & 220 °C for 15 min | 109 | 0.01% | Ali et al. (2012a, 2012b) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Simplex qPCR (Molecular Beacon) | Pork | Cytb | Normal | 119 | 0.0001 ng (pure) & 0.1% (admixture) | Yusop et al. (2012) |
| Multiplex qPCR (TaqMan) | Chicken, pig and duck | Transforming growth factor, beta actin & T cell growth factor | Normal | 76, 111 & 212 | 0.15 ng (pure) & 1% (admixed) | Cheng et al. (2014) |
| Multiplex qPCR (TaqMan) | Pork and beef | Cyclic-GMP-phosphodiesterase & Beta-actin | Normal | Not given | 20 genome equivalents | Iwobi et al. (2015) |
| Multiplex qPCR (TaqMan) | Beef, buffalo and pork | ND5 & cytb | Normal | 106, 90 & 146 | 0.003 ng (pure) & 0.1% (admixed) | Hossain et al. (2017) |
| Droplet digital PCR | pig, cattle and horse | F2 | Normal | Not given | 0.001% | Floren et al. (2015) |
| Droplet digital PCR | Pork and chicken | Beta-Actin & TGFB3 | Normal | Not given | 40 & 80 ng | Cai et al. (2014) |
| Duplex droplet digital PCR | Beef and pork | Beta-actin | Normal | Not given | ~0.1 ng/μL | Cai et al. (2017) |
| Droplet digital PCR | Porcine, bovine, chicken and turkey | ATPase, ATP synthase, Cytb and 12S rRNA, | Autoclaved | Not given | 0.01–3.0% | Shehata et al. (2017) |
| DNA barcoding | Beef, chicken, lamb, turkey, pork and horse. | COI | Normal | 658 | – | Kane and Hellberg (2016) |
| DNA barcoding | Poultry | COI | Heavily processed products | 658 and 127 | – | Hellberg, Hernandez, and Hernandez (2017) |
| DNA barcoding | Beef, chicken, lamb, goat, buffalo, pork, duck, prawn and fish. | COI | Chefs and cooking | ~650 | – | Ahmed et al. (2018) |
| Biosensor | Pork | Cytb | Autoclaved for 2.5 h | – | 0.5% | Ali et al. (2011) |
| Biosensor | Pork, chicken and bovine | Cytb | Normal | – | 20.33-78.68 pg/μL | Ahmed et al. (2010) |
| Biosensor | Pork | Cytb | Normal | – | 0.58 μg/mL | Hartati et al. (2019) |
| DNAFoil technique | Pork | – | Normal | – | 0.1% | Meat and Livestock Australia Limited (MLA) (2018) |

amplifying 609 bp products from five most commonly consumed animals namely pig, cattle, goat, buffalo, and sheep. The amplified PCR products were digested with *Alu*I and *Taq*I REs which resulted in distinctive digestion maps able to discriminate each species. The developed method is suitable to distinguish the meats of closely related species of domestic livestock. Similarly, PCR-RFLP technique was applied for differentiating seven species such as pork, goat, beef, buffalo, chicken, rabbit and quail targeting 359 bp mitochondrial cytb gene. To differentiate the meat species *Alu*I, *BsaJ*I, *Rsa*I, *Mse*I, and *BstU*I enzymes were selected which generated individual restriction pattern for each species (Murugaiah et al. 2009). Another porcine specific PCR-RFLP method was developed for the authentication of meat and meat products. The method was highly efficient because there was no adverse effect of heat, processing treatments and used ingredients on the amplification of the PCR products. The designed primer set amplified 276 bp PCR product from pig mitochondrial D-loop region and amplified products were further ensured by digestion with *Hha*I and *Sau*3AI REs. The developed technique was able to detect porcine material up to 1% adulterated and autoclaved admixed samples (Mane, Mendiratta, and Tiwari 2013). Rahman et al. (2014) approached dog specific PCR assay targeting 100 bp fragment of mitochondrial cytb gene. The sensitivity of the method was 0.2% in ternary admixture under extensively autoclaved samples. Rashid et al. (2015) designed a set of macaque-specific primers which amplified 120 bp fragment from D-loop gene of monkey species and the distinctive RFLP patterns were generated by digestion with two REs (*Alu*I and *CVi*KI-1) to authenticate the PCR products. PCR assay was also documented for the identification of dog, cat, horse and donkey and the originality of the amplified fragments were confirmed by RFLP analysis (Abdel-Rahman et al. 2009).

A very short-length targeted PCR method applying lab-on-a-chip-based automated electrophoretic system was developed for the detection of feline species in food. The developed system amplified 69 bp PCR products and the amplified fragment was authenticated by digestion with *Alu*I RE. Superiority of the short-targeted biomarker over long targeted one was also proved based on sensitivity and stability. The two digested fragments (43 and 26 bp) of RFLP fingerprint were visualized in both the gel-image and electropherograms. The LOD of the assay was found to be 0.01% (w/w) for feline meat in binary and ternary admixtures as well as in meatball matrices indicating that the method could be applicable for the detection of feline DNA in severely degraded samples (Ali et al. 2015a). Similarly, Malayan box turtle (MBT) was detected using lab-on-a-chip-based PCR- RFLP method targeting 120 bp fragments from cytb gene. The amplified products were digested with *Bfa*1 RE and the LOD was found 0.0001 ng DNA and 0.01% under raw and admixed samples respectively (Asing et al. 2016b).

Like simplex PCR assays, several mPCR assays have been reported for the detection of multiple species in a single assay reaction. A common primer mPCR was developed for the simultaneous detection of four species, namely pig, cattle, chicken, and horse. Matching with the DNA sequences for each species, a common adapter was designed in the 5′-end of species-specific reverse primers and it was used as the common primer which amplified 412, 292, 239 and 451 bp fragments from pig, cattle, chicken and horse respectively. The sensitivity of the system was found to be 0.1 ng DNA. The use of common forward primer for all target species offers more advantages over conventional mPCR systems by reducing the competition among primers in the reaction mixture resulting in increased specificity and sensitivity (Bai et al. 2009). Nejad et al. (2014) developed an mPCR assay for the simultaneous detection of five species (chicken, goat, cow, camel and donkey) targeting 183, 157, 274, 200 and 145 bp respectively from mitochondrial 12S rRNA, cytb and NADH dehydrogenase subunit 2 genes. Although they found the sensitivity of the simplex assays up to 0.05 ng for each species, they could not determine the specificity and sensitivity of the mPCR system. Zhang (2013) introduced another type of mPCR system following two strategies; applying semi-nested mPCR and shortening the number of primers to enhance the sensitivity of mPCR. They used a pair of common primers to perform the first PCR assay followed by the use of amplified product as the template of second mPCR. This method was very effective to detect meat species in processed foods increasing the sensitivity of the mPCR assay by 3-fold compared to conventional system. The LOD of semi-nested mPCR was found to be 1 pg for simultaneous detection of pork, chicken, beef and mutton species. Another rapid and cost-effective mPCR method was successfully developed for the first time without requiring the extraction of DNA. The method was able to identify six common species like pork, beef, chicken, lamb/mutton, ostrich meat, and horse meat in highly degraded and processed food samples with the detection limit of 7 fg (Kitpipit, Sittichan, and Thanakiatkrai 2014). To identify pig, cattle, poultry (chicken and turkey), and equine (horse and donkey) species simultaneously, mPCR was applied by Ilhak and Guran (2015). The market survey of 50 sausage samples (beef and poultry) revealed that the developed assay successfully identified 23.3% poultry adulteration in beef sausage samples and equines were positive for 2% samples, but porcine material was absent in all tested samples. Another mPCR was developed for simultaneous detection of five meat species that are not considered as Halal food. Five pairs of species-specific primers were designed targeting mitochondrial ND5, ATPase 6, and cytb genes to amplify 172, 163, 141, 129 and 108 bp DNA fragments from cat, dog, pig, monkey and rat meats respectively. The LOD of the assay was 0.01–0.02 ng DNA under raw state and 1% meat adulteration in meatball formulation under raw and heat treated (121° C for 2.5 h) states (Ali et al. 2015c). A hexaplex PCR was developed for detecting simultaneously five meat species namely pig, cow, poultry, sheep and horse as well as one plant species such as soybean. The assay sensitivity was found to be 0.01% in complex matrices (Safdar and Junejo 2016). Multiplex PCR assay was also developed to discriminate rat, rabbit and squirrel meat in food products (Ahamad et al. 2017). More recently, heptaplex PCR

assay was developed for the detection and differentiation of cow, buffalo, chicken, cat, dog, pig and fish species simultaneously targeting short amplicon length (73 – 198 bp). The sensitivity for raw meat was found as 0.01 – 0.001 ng of DNA and 0.5% (w/w) meat, for meatball products (Hossain et al. 2019).

Recently, an innovation was introduced for the animal species authentication of food products by developing double genes targeted multiplex PCR-RFLP assay. Very short-length biomarkers (73-146 bp) were designed for this novel multiplex PCR-RFLP technique targeting two different sites of mitochondrial conserved regions (cytb and ND5 genes) for each of the bovine, buffalo, and porcine materials. Better security was obtained with this authentication in two ways; first through a complementation strategy as it is highly unexpected that both sites will be lost under compromised conditions, and the second way is through molecular fingerprints. Cross-amplification reaction and restriction digestion of PCR products with *Alu*I, *Eci*I, *Fat*I, and *CviKI*-1 enzymes provided target specificity and authenticity. Finally, the developed assay was tested with commercially available frankfurters and it was observed that the obtained results varied with the actual labeling. Thus, this new approach can be applied as a reliable tool for identification of animal sources in commercially available Halal and Kosher food products (Hossain et al. 2016). Later, another double gene targeting mPCR was documented for the detection of crocodile DNA in commercial products namely, crocodile jerky, crocodile soup, spicy crocodile meat, crocodile herbal soup and over-the-counter crocodile balm. The method was validated under samples with various heat-treatments namely, boiling, microwave cooking and autoclaving as well as under binary admixture. The assay was highly stable under all treatments and successfully amplified both targets up to 1% (w/w) adulterated meat (Ahmad Nizar et al. 2018).

Recently, researchers are more interested in using fully automated multi-capillary electrophoresis device instead of conventional agarose gel electrophoresis to separate and visualize the amplified PCR products. Conventional gel electrophoresis cannot clearly distinguish DNA fragments of less than 50 bp length difference whereas, the capillary system effectively enhances sensitivity and resolution (~5 bp length difference) saving analysis time and also minimizing the manual handling errors. In addition to gel images, it also clearly indicates the PCR product size through electropherograms (Hossain et al. 2017a).

Although PCR based methods became a popular technique for the authentication of meat products due to its stability, specificity and sensitivity, however, PCR amplification could be affected by food ingredients such as fats and proteins (Raja Nhari et al. 2016). This limitation can be overcome by modifying DNA extraction method making it more suitable to extract highly purified DNA from the food products.

### Quantitative real-time PCR (qPCR)

Due to inability of the EP-PCR to provide quantitative information of the target species originally present in the specimens, researchers have been paying increased attention to automated real-time PCR (qPCR). In contrast to conventional PCR assays, qPCR techniques are especially promising because of full automation, rapidity and high sensitivity. In addition to detection, they also offer quantification opportunities of the analyte targets at real-time, eliminating the time-consuming post PCR analysis step like electrophoresis (Asing et al. 2016a; Cheng et al. 2014). Particularly, qPCR involves direct monitoring of generation of PCR products during each amplification cycle and can measure at the exponential phase of the reaction where there is no need to complete the reaction. Unlike EP-PCR assay, this system allows quantification of the PCR products at an initial stage of the reaction with more precision and accuracy. As fluorescent molecules are used to collect the real-time data, there is a high correlation between intensity of the fluorescent dye and the quantity of PCR products (Agrimonti et al. 2019; Fajardo et al. 2010). Two general categories of fluorescent chemistries namely double-stranded (ds) DNA-intercalating dyes such as SYBR Green (Asing et al. 2016a) or Eva Green (Safdar and Abasıyanık 2013) and probe-based chemistry such as TaqMan (Ahamad et al. 2019; Ahmad Nizar et al. 2019) or Molecular Beacon (Hadjinicolaou et al. 2009) probes are available for the qPCR systems. The main drawback of DNA-intercalating dye chemistry is that it nonspecifically binds any dsDNA including primer-dimers that are available in the reaction tube making the detection false positive and unreliable (Arya et al. 2005). Moreover, some dyes are known to inhibit the PCR reaction (Gudnason et al. 2007). In contrast, TaqMan probe-based technique is widely acceptable since both the primers and probes find their complementary sites in the template DNA and thus offer the chance of double checking which increases specificity and reliability of the technique (Hossain et al. 2017b). After hybridization of the specific probe, fluorescent signal is generated as the DNA polymerase moves by and cleaves off the probe's reporter and quencher molecules. In addition, TaqMan probe techniques are advantageous in developing multiplex qPCR (mqPCR)systems, because labeling of specific probes can be done with different reporter dyes that allow the identification of amplified targets formed by single or multiple primer sets in one assay tube (Arya et al. 2005). Hence, unlike singleplex qPCR, mqPCR could be advantageously applied for detection and quantification of multiple target oligos in one platform, which can save both analytical time and cost (Hossain et al. 2017b; Iwobi et al. 2015). The various stages in the development of qPCR are given in Figure 2.

The qPCR methods have been used for the identification and quantification of animal species in various Halal and Kosher meat and meat products (see Table 3). A rapid qPCR assay was developed for the detection of trace amount of pork, beef, mutton, chicken and horseflesh in processed foods. Here, cytb gene was targeted to design the primers and TaqMan minor groove binder (MGB) probes. The limit of quantification of this method was found to be 0.0001% (10 fg/μL) of each species for pure state (Tanabe et al. 2007). A heptaplex qPCR has been reported to quantify the

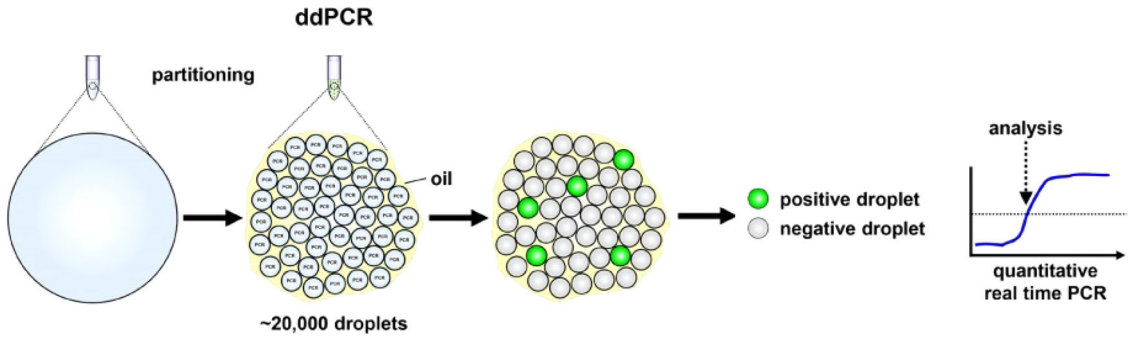


**Figure 3.** Schematic model of droplet digital and quantitative real-time PCR. Droplet digital PCR (ddPCR) reaction reagents are partitioned into ~20,000 droplets before PCR reactions proceed to the reaction plateau end point in individual droplets. Droplets are assessed as positive or negative from their fluorescent signal intensity (reprinted from Lodrini et al., 2017, licensed under CC BY 3.0).

fractional proportion of pork, beef, goat, sheep (mutton), chicken, turkey, and horse meat simultaneously. The assay sensitivity was 2% under admixed DNA (Köppel, Zimmerli, and Breitenmoser 2009). The qPCR assay was improved targeting shorter DNA fragments (109 bp) from cytb gene for the detection and quantification of porcine material in meatball and burger formulations using TaqMan probe. The developed method can detect upto 0.01% pork adulteration in beef meatball and burger under cooking and grilling (Ali et al. 2012).

Furthermore, to increase the assay specificity, Yusop et al. (2012) introduced qPCR assay for the detection of pork using molecular beacon (MB) probe from cytb gene targeting 119 bp amplicon. The LOD was found to be 0.0001 ng DNA for pure state and 0.1% (w/w) pork in pork–beef admixture. It is known that MB enhances the sensitivity and specificity of the assay since it can identify single nucleotide polymorphism (SNP). MBs are hairpin-shaped oligonucleotide probes which consist of a stem and loop with fluorescent reporter dye at 5′end and quencher dye at 3′end. These two dyes are in close contact with each other at the end of the stem of the hairpin. The loop consists of 15-20 nucleotides which are complementary to the target sequence. This facilitates the MB-based assay to amplify only target species by eliminating the chance of nonspecific binding. The Tm values of the primers as well as stem and loop region of the probe should be kept in a range which is suitable for successful amplification (Mohamad et al. 2018; Stratagene 2010).

A triplex TaqMan probe-based qPCR assay was reported to identify pig, chicken and duck in blood curds. Total DNA from blood curds samples was extracted using three different kits namely TIANamp®Blood DNA Kit, phenol/chloroformextraction method and TIANamp®GenomicDNA Kit to compare DNA yields and purity by measuring the concentration of DNA and the ratio of the absorbance at 260 and 280 nm. Better efficiency was found for the TIANamp®GenomicDNA Kit and modified phenol/chloroformextraction method compared to TIANamp®Blood DNA Kit. The sensitivity of the assay was 0.15 ng under pure state (1:103 dilution) and 1% under ternary admixture for each target species (Cheng et al. 2014). Another triplex qPCR assay was approached for the quantification and differentiation of pork and beef in minced meat products. In this technique, pig and cow specific primers and probes were employed against mammals and poultry species specific myostatin universal system. The sensitivity of the qPCR assay was 20 genome equivalents with the measurement of uncertainty at 1.83% and the method showed good reproducibility and robustness under validation on several commercially available minced meat products (Iwobi et al. 2015). A tetraplex qPCR assay with TaqMan Probes was developed for the quantitative detection of pig, cattle and buffalo. To avoid ambiguity in molecular diagnostics because of breakdown of target DNA, the amplicon sizes were kept very short (90-146 bp). To eliminate the false negative detection, 141 bp eukaryotic 18S rRNA endogenous control was used as internal amplification control (IAC). The LOD of the developed system was 0.003 ng under pure state and 0.1% under ternary admixture with $84-115\%$ target recovery for three target species (Hossain et al. 2017b). More recently, Khairil Mokhtar et al. (2020) reported a porcine specific qPCR assay to establish a rapid, efficient, cost-effective, and simple DNA extraction technique which is useful for both raw and processed meat products. They tested three different formulations of lysis buffer namely LB1 (5% Chelex-100 in water suspension), LB2 (0.2 M Tris-HCl, 0.01 M EDTA, 0.5 M NaCl and 1% SDS), and LB3 (5% Chelex-100 in LB2 suspension) to extract the DNA from meat samples. They evaluated the effect of lysis buffer on quality and quantity of the extracted DNA as well as on the sensitivity and specificity of qPCR assay. The LB3 buffer was found to be the most effective for high quality DNA extraction for successful qPCR assay.

Another new approach in qPCR technology is droplet digital PCR (ddPCR) which offers accurate quantification with improved precision and sensitivity and eliminates the effect of complex matrices thereby providing an absolute measurement of DNA concentration without the use of standard curves. With the help of Poisson distribution law, the fraction of positive droplets can be converted to the number of molecules in the starting sample where no standard curves are required at all (absolute quantification). Here, each reaction is randomly distributed in several thousand nanoliter-scale water in oil droplets. The total number of target molecules initially present in the experimental sample can be measured accurately by the ratio of positive to total number of droplets (Morisset et al. 2013). Therefore, low

concentrated DNA templates in the mixture of higher numbers of non-target DNA can be detected using ddPCR and hence, a direct relative quantification is possible (see Figure 3) (Floren et al. 2015). Floren et al. (2015) described an authentic two-step ddPCR assay for the quantification of pig, cattle and horse species targeting the F2 gene in processed meat products. Herein, nuclear gene was used instead of mitochondrial gene to overcome the quantification problem with mt-DNA particularly in processed food because it may contain fat, offal and tendon in addition to muscle meat and thus, it is sometimes difficult to quantify the trace amount using mitochondrial genes. In fact, for quantification based on nuclear DNA, it is not necessary to have knowledge of tissue type and it is believed that the result can be expressed as genome/genome equivalents and not on weight/weight. They found that ddPCR assay was more advantageous than qPCR showing the LOD and LOQ in meat products of 0.001% and 0.01% respectively. Cai et al. (2014) developed a highly precise ddPCR method for pork and chicken for the detection and quantification of low copy number template DNA, as in some complex mixture meat products. By utilizing the ddPCR, a relationship was established between the weight of raw meat and the weight of DNA as well as between the weight of DNA and copy number DNA which were both close to linear. This helped to develop a formula for calculating the raw meat weight based on the DNA copy number. The method was validated in terms of accuracy and applicability under 10-90% binary admixture of pork and chicken. The sensitivity of the system under pure state was found to be 40 and 80 ng DNA for chicken and pork, respectively. In 2017, the same group also developed a ddPCR based duplex PCR assay for the detection and quantification of the beef and pork in a single reaction. The LOD and LOQ of the duplex system were ~0.1 ng/μL and 0.5 ng/μL respectively for both targets (Cai et al. 2017). Another ddPCR assay was developed and evaluated targeting mitochondrial genes for identification and quantification of porcine, bovine, chicken and turkey DNA in food sample. An artificial recombinant plasmid DNA cloned with 134 bp artificial DNA fragment, was used as IAC to normalize variabilities in the PCR reaction, to protect any false negative amplification as well as to assure reliability of the assay. The quantification ranges were 0.01 ± 1.0% (wt/wt) for pork and chicken, and 0.05 ± 3.0% (wt/wt) for bovine and turkey under fortified heat-processed food products (Shehata et al. 2017).

### DNA barcoding

Among the available techniques in species detection, DNA barcoding is one of the most promising candidates with high accuracy and efficiency for differentiating meat of various animal species. DNA barcoding is a sequencing-based method and involves COI gene targeted biomarker which amplifies approximately 650 bp fragment through PCR reaction. Mitochondrial COI gene has been appropriate for species discrimination because it shows relatively high level of sequence divergence between species and low level of divergence within species. Compared to several other available techniques, DNA barcoding seems to be more time-consuming, but it has an extra advantage of allowing for a comprehensive approach for species detection favored by a high level of genetic information. Moreover, this technique can easily support high-throughput automation (Hellberg, Hernandez, and Hernandez 2017). However, animal meats in food products are usually processed through boiling, canning and stir-frying which can cause degradation of DNA, resulting in difficulties for the amplification of target fragment of full-length DNA barcodes. To overcome this limitation using full-length barcode, researchers have paid more attention to the mini-barcoding technique where sequence of target length is reduced by targeting a shorter fragment within the standard barcode region, improving the amplification possibility and capability. A number of recent studies have documented successful amplification and sequencing of a variety of mini barcodes from different processed products. Thus, both full length DNA barcoding (Ahmed et al. 2018; Haye et al. 2012; Hellberg, Hernandez, and Hernandez 2017; Kane and Hellberg 2016) and mini barcoding (Hajibabaei et al. 2006) techniques have been widely applied for Halal and Kosher authentication (see Table 3).

### Biosensors

Nowadays, one of the key achievements in nanomaterial science and nanobiotechnology is the development of novel sensors and biosensors for food analysis. Given their simplicity, rapidity, portability, and low-cost, they are considered as potential tool in food safety issues (Gooding 2002; Pérez-López and Merkoçi 2011). A typical DNA biosensor can be developed by immobilizing short-length single-stranded DNA probe, also called nucleic acid recognition layer on to the surface of the nanomaterials (signal transducer). The role of the immobilized DNA probe (nucleic acid recognition layer) is to detect specific complementary target DNA sequence by hybridization. This hybridization can be determined by signal transducer and transduction of hybridization of template DNA can be measured by the signal intensity of the fluorescence dye attached to the probe DNA. The transducer can be electrochemical, optical or piezoelectric. Thus, two main steps are involved in the development of biosensor; first, immobilization of target DNA probe to the transducer (e.g. SiC, GaN/GaAlN, AlN, ZnO, Au and Diamond) and second, signal transduction (Figure 4) (Gooding 2002; Sassolas, Leca-Bouvier, and Blum 2008; Wang et al. 2015).

Over the last few years, nanotechnology has evolved as one of the most promising tools regarding food safety and food integrity issues for various purposes like detection of contaminants, making the preservatives to be released in a controlled way to increase shelf life of foods, and improvement of food-packaging strategies. To design sensors and biosensors for food analysis, nanomaterials like metal oxide and metal nanoparticles, carbon nanotubes, and quantum dots play a vital role (Inbaraj and Chen 2016). There are many advantages offered by gold nanoparticles (GNPs) in

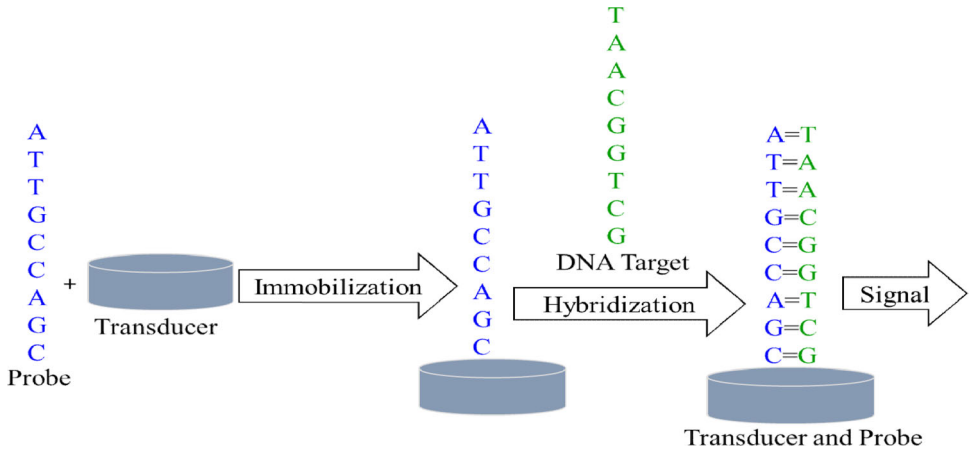


**Figure 4.** Steps involved in the detection of a species specific DNA sequence using an electrochemical DNA hybridization biosensor (adapted from Gooding 2002). 

sensory research. Firstly, GNPs are nicely compatible with almost all types of chemically (organic and metallic) and biologically active molecules. In other words, these molecules can remain functionally active even after they are immobilized on GNPs. Secondly, GNPs can help to immobilize large quantities of organic or biomolecules, as they have high surface area to volume ratios which in turn may increase the chances of their interaction with a target analyte. Thirdly, GNPs (e.g., citrate capped) are mostly negatively charged which made them suitable for electrostatically interaction with certain positively charged biomolecules thereby making the future possibility of highly selective interaction exclusively with the target analyte of interest. Moreover, one advantage of biofunctionalized GNPs is its multiplexing in the detection of analytes. In multiplexing, GNPs hosting multiple ligands can interact with multiple receptors selectively and simultaneously which make it possible to detect multiple target analytes at the same time (Upadhyayula 2012). The biosensor-based detection techniques are summarized in Table 3.

A novel nanobiosensor was introduced by integrating a 27-nucleotide *Alu*I digested fragment of pig mitochondrial cytb gene to a 3-nm diameter citrate-tannate coated GNP that can detect porcine DNA in admixed autoclaved meat products in a single step excluding the separation or washing steps. Here, synthetic swine specific short-length oligonucleotide was labeled with an alkanethiol at one end and tetramethyl-Rhodamine (TMR) fluorescent dye at the other. Citrate-tannate-coated GNPs were prepared in a diameter of 3 nm. The labeled oligo was self-organized into an arch-like closed conformation when bonded to the GNP surface via sulfur-gold bond, hence the fluorescence was almost-completely absorbed by the nanoparticle through nonradiative energy transfer. Due to hybridization of the target DNA to oligo probe, the constrained structure took into a straight conformation resulting in the release of fluorophore from the surface of the GNPs. The produced fluorescence signal due to conformational changes of the GNPs was highly specific and sensitive to the target DNA binding and could successfully detect the porcine materials up to 0.5% in raw and 2.5 h autoclaved binary admixture (Ali et al. 2014a).

Ahmed et al. (2010) approached a new method for the differential detection of raw and processed pork, bovine and chicken meats based on loop mediated isothermal amplicons (LAMP) and disposable electrochemical printed (DEP) chips. Here, LAMP was a target DNA amplification method that amplified nucleic acid in isothermal condition (63° C) with high efficiency, specificity and rapidity. By using electrochemical genosensors, amplicons were detected by DEP (disposable electrochemical printed) with the help of Linear Sweep Voltammetry (LSV) through observing DNA–Hoechst33258 [2-(4-hydroxyphenyl)-5-(4-methyl-1-piperazi-nyl)-2, 5-bi (1H-benzimidazole), H33258] interaction on the chip surface. Hoechst33258 interacts with DNA in solution where immobilization of DNA onto the electrode surface is not required and the time for probe immobilization step is saved. This method is more sensitive and specific while avoiding unexpected amplifications compared to mPCR assay and LOD was found to be ~20.33 ng/μL ($3 \times 10^4$ copies/reaction), ~78.68 pg/μL ($3 \times 10^2$ copies/reaction) and ~23.63 pg/μL (30 copies/reaction) for pork, chicken and bovine species respectively. More recently, gold-modified screen-printed carbon electrode (SPCE-Gold) based electrochemical DNA biosensor technique was reported for the detection of porcine DNA. Herein, bio-conjugates were created by immobilization of the pig mtDNA probe with gold nanoparticles of the SPCE-Gold using differential pulse voltammetry method. Moreover, voltammetry based on methylene blue indicator signal was applied to characterize the hybridization of the target DNA to DNA probe (see Figure 5) (Hartati et al. 2019).

### *DNAFoil technology*

DNAFoil technique is a rapid, on-site, simple, and self-contained DNA test which requires about 30 min to detect the species without involving costly PCR instrument or laboratory setup. The method is very simple comprising of five steps (Figure 6). Typically, sample is crushed with a barrel

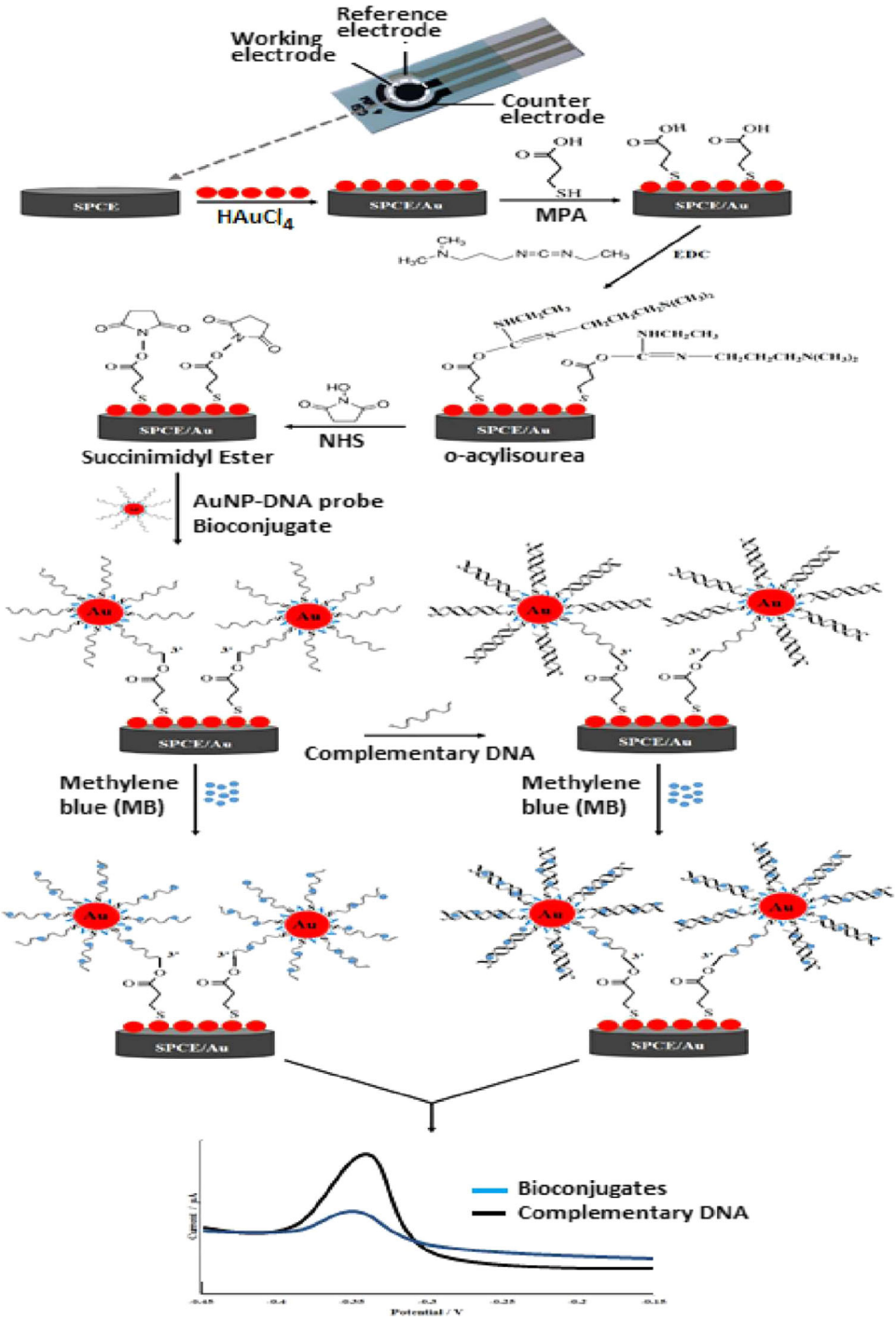


**Figure 5.** Overall scheme of DNA biosensors based on AuNP–DNA probe bioconjugates on the SPCE-Gold surface, with hybridization detection using methylene blue (reprinted from Hartati et al. 2019, licensed under CC BY 4.0 http://creativecommons.org/licenses/by/4.0/. No changes were made in the reprinting of the figure.).

and then mixed with hot and cool water. Herein, cell lysis and DNA extraction are accomplished without centrifuges and spin-columns. To amplify the target DNA, one drop of extracted DNA is transferred to the specific reaction tube and incubated in water bath. The reaction tube containing target specific primers and enzymes involves amplifying the target DNA as well as producing multiple copies within 30 min. The target DNA amplification is carried out in one tube master mix without using thermal cycler and temperature gradients. Then, revealing test strip is inserted in the reaction tube, the strip material allows the target DNA to carry away through the identification surface using capillary force and this helps to facilitate the target DNA to hybridize with specific complimentary captured probe sequences. This hybridization allows to visualize the results as a bead by naked eyes due to color change. Thus, the method offers instant, low cost, easy to interpret and on-site detection eliminating the use of expensive equipment (El Sheikha 2019).

Meat and Livestock Australia Limited developed DNAFoil kit for the detection of pork. The kit comprised of three components, including preparation barrel, reaction

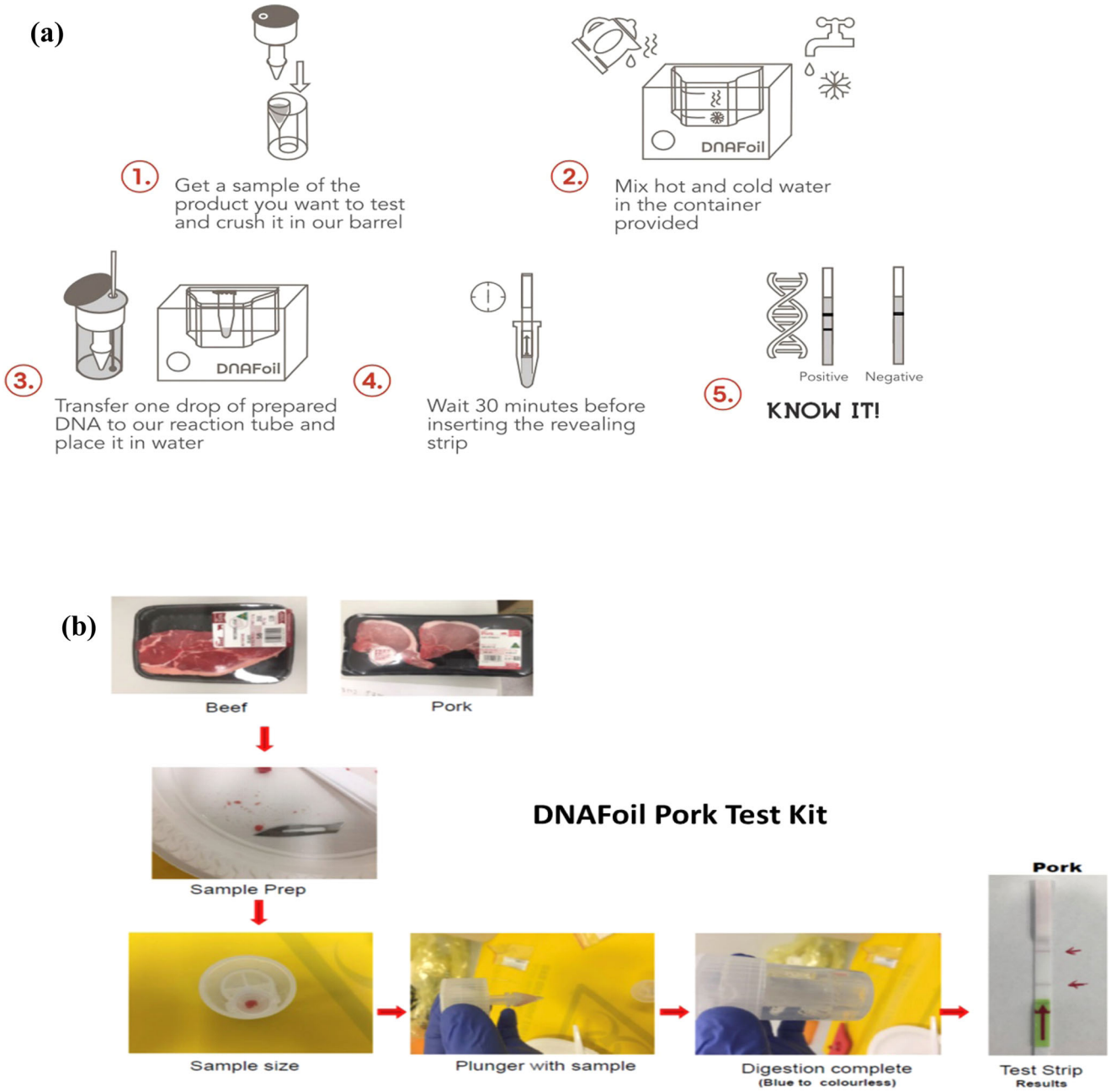


**Figure 6.** (a) The procedural mechanism of DNAFoil technique. (b) The flowsheet of sample preparation and digestion using DNAFoil Pork Test Kit (reprinted from El Sheikha 2019). © 2019 Aly El Sheikha. All Rights Reserved. Reproduced with permission from Elsevier.

mixture tube and test strips. The negative and positive results were indicated with the visualization of single and double lines on the strip, respectively. The kit was validated under pure and binary admixtures of 1% and 0.1% pork contamination in beef matrices. A single line was visualized on the strip when pure (100%) beef was used as starting sample resulting negative for pork and the reaction is completed successfully. While pure pork was used as positive control, double lines were observed on the strip. Similarly, double lines were also found in the case of 1% and 0.1% pork adulterated samples indicating positive result for pork. However, despite all preventive measurements against cross contamination, the kit showed false positive for pork when pure beef was used as negative control. Thus, the system failed to prove its robustness (Meat and Livestock Australia Limited (MLA) 2018).

## Spectroscopic methods

Spectroscopic methods have also been considered as reliable tools for the authentication of Halal and Kosher products. These techniques help analyzing characteristics or properties of materials through the use of radiated energy. Herein, intensity is measured as a function of wavelengths and spectrums are generated for comparative analysis. These techniques involve interaction of light with matter and thus by investigating several features of a given sample, they provide information about its consistency or structure. Light is considered as electromagnetic radiation and this phenomenon

exhibits a variety of energies based on which molecular features could be investigated (Hofmann 2010). For example, Fourier transformed-infrared (FT-IR) spectroscopic method was applied for the detection of pork in binary admixture of buffalo or camel meats (Lamyaa 2013), lard in meatball broth (Kurniawati, Rohman, and Triyana 2014), pork in beef meatballs (Rohman et al. 2017), pork in ground beef meat (Hu et al. 2017) and pork in beef and mutton (Yang et al. 2018). Near Infrared (NIR) spectroscopic approaches have been reported to differentiate between fresh and frozen–thawed pork samples (Barbin, Sun, and Su 2013) and repeatedly frozen pork (Huang et al. 2016). Similarly, VIS-NIR based method was reported to detect pork, chicken, beef and lamb (Cozzolino and Murray 2004), llama, cattle and horse (Mamani-Linares, Gallo, and Alomar 2012) and to discriminate fresh and frozen-thawed pork (Pu et al. 2015). FT-NIR based technique was also applied for the detection of pork adulteration in the veal sausage (Schmutzler et al. 2015).

## Food additives

According to Codex, "*Food additive means any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities*" (Alimentarius 2015). The numerous organic compounds which are edible substances are used as food additives. The role of these compounds might be as preservatives, flavoring agents or colorants. Preservatives are usually used to preserve meat and other products. The main reason to use preservatives is to protect foods from the influence of biological factors (microorganisms), to maintain normal condition and to make appear fresh much longer. The flavor additives play an important role to complement, enhance and modify the test and aroma of the food products. The flavor and taste of food help stimulating salivary flow and acid digestion. The color additive is any kind of pigment, dye or substance which is able to give color upon addition to food products.

The methods for the analysis of food additives depend on the nature of chemical compounds present. Chromatographic techniques are one of the most important methods for the separation and differentiation of organic compounds used as food ingredients. These methods can be applied with high separation efficiency for the analysis of food components to detect trace amounts of food additive and pesticide residues. Therefore, GC, HPLC, GC-MS and LC-MS based methods may be applicable in such cases where organic compounds like preservatives, flavoring agents or colorants are concerned (Ballin 2010; Rahmati et al. 2016). Other types of additives are enzymes which are involved in blood clotting. By using as blood-based binding agents these can be added to meat cuts or minced meat to make a definite mass and shape. Here, fibrinogen is cleaved into fibrinopeptides A and B by the action of thrombin. Bovine (Grundy et al. 2007) and porcine (Grundy et al. 2008) fibrinopeptides A and B can be detected in concentrations down to 5% by using LC-MS/MS. The methods can differentiate between blood-based binding agents of bovine and porcine origin because fibrinopeptides A and B are species-specific (Ballin 2010)

To perform safety assessment of food additives, it is imperative to review all available toxicological data covering observations in humans and in animal models. From this data, a maximum level of an additive known as 'no-observed-adverse-effect level' (NOAEL) is then determined which does not show any apparent toxic effect. The 'Acceptable Daily Intake' (ADI) figure for each food additive is established by using it. The ADI indicates the amount of a food additive that is allowed daily in the diet for a long time, even over a lifetime period, having no adverse effect on health (Alimentarius 2015; Eufic 2004).

## Prospects and challenges

Analytical testing plays a vital role in Halal and Kosher authenticity issues. Remarkable progress has been made in the specificity and sensitivity of analytical methods. Based on requirements, each technique is complementary to the others providing its own limitations and advantages. As stated earlier, the protein-based techniques are not completely reliable for authentication of species, particularly in highly processed food and when a lower detection limit is a requisite. Moreover, this method is also not suitable for the differentiation of species in mixed matrices especially in closely related species due to occurrences of cross-reactions. DNA based techniques, however, are more reliable and stable with high specificity and sensitivity offering discriminatory power of identifying even closely related species under complex matrices, saving both time and cost (Ali, Razzak, and Hamid 2014b; Sahilah et al. 2012). In contrast to singleplex assay, the major challenge in the development of mPCR assay is the design and optimization of biomarkers, since all targets are amplified in a single reaction mixture and all primers should have very close Tm as mPCR efficiency is affected by little differences of Tm between the primer sets. Another critical step involves in maintaining all the amplicons (target sites) very short ($\geq$ 200) because longer targets could be broken down under severe food processing conditions (Ali et al. 2015a). Thirdly, availability of appropriate probe reporter dyes as well as detector system which can detect assigned dyes simultaneously in case of real-time PCR assay. The recent innovation in the mPCR assay targeting double genes instead of one can be a new pathway for detecting animal and gelatin sources in commercially available Halal and Kosher food products. This novel mPCR assay is outstanding because it ensured extra security through targeting two different sites of two different

genes that are less likely to break down even under the states of decomposition. The species-specific PCR-RFLP assays are especially interesting because they offer the opportunity to authenticate a product by restrictive digestion of the amplified PCR products using one or more REs and capable of differentiating even closely related species.

The advanced analytical systems, now a days, are considered as suitable tool to fulfill the global requirement on Halal and Kosher meat products' authenticity. Although the PCR-based techniques have been proved as reliable and authentic tools in food quality and safety issues, their practical application at the industrial level is still limited due to lack of rapidity, simplicity, and field-based support. Hence, there is a need of portable, fast, low cost and simple tool for the detection of species in food products. The demand could be fulfilled with the use of DNAFoil technology which is a rapid, portable, and on-site DNA analytical tool. In addition, commercial kits are also available to support the Halal and Kosher authentication issues. However, they are not suitable for the highly processed food products since protein is denatured easily by processing treatments.

Currently, researchers have paid more attention in the nanotechnology-based biosensor and microarray systems for the detection of DNA and these approaches have received enormous responses from different sources such as industries, funding organizations and research institutes. These detection techniques gained popularity as they are fast, cost effective and highly sensitive and do not require expensive laboratory set up, costly instruments and skilled personnel and on-field delivery of results can also be developed. However, identification of DNA by using sensor devices may face some major challenges. First, as these devices still need to be further developed, the use of most of these sensors are limited in the laboratory scale and the tests are done with purified DNA thereby requiring its extraction from a biological specimen. Thus, on-site delivery of results is one of the most important issues to be considered in future. Second, due to use of surface-tethered probe DNA in these devices, the flexibility in surface reaction in target hybridization becomes limited. Conformation of the nucleic acid and freedom of movement on the surfaces are greatly disturbed by the substrate, conjugation chemistry, and grafting density. In addition, there is a tendency of DNA which is bound to the surfaces, to take on a flat conformation. As a result, the phosphate backbone or the hydrophobic bases can strongly interact with the substrate surface and the base-to-base interaction which is useful for the specific recognition of the incoming targets becomes restricted. Although there is a greater control over the orientation due to covalent attachment caused by end grafting or affinity coupling, the nucleic acid chains try to assume an extended conformation. However, recently diamond quantum dots have been introduced to minimize these limitations for the detection of DNA targets in a solution-like environment. The third challenge seems to be the crowding or steric hindrance which is very commonly observed in probe target interactions onto the surface-tethered immobilized probes and this can be minimized by using a suitable linker in the immobilized probe. The fourth challenge might be the frequent occurrence of nonspecific interactions because of cross hybridization of the probe with the target, and nonspecific adsorption of the target onto the surface. The possible way to reduce nonspecific interactions might be the use of suitable blockers, a specifically designed probe, optimization of the salt concentration and hybridization (Ali et al. 2018; Švorc et al. 2015).

## Conclusion

The religious certification agencies have been performing a crucial and complicated job to follow all the ingredients throughout their different manufacturing stages, to disclose the full history, i.e., full traceability, of the ingredients and products to certify them. Thus, for certifying products, a religious certification agency needs to establish the standards along with its trademarked symbol on it, and at the same time, it must be technically competent to provide a true assurance that its standards are maintained. The application of laboratory authentication techniques can then provide a supplementary role on the-ground activities to maintain the integrity of the whole approach as it is almost impossible to ensure continuous supervision for a huge variety of materials circulating into the Kosher and Halal chain. In addition, it is well known that testing alone is not enough, and it only assists in reinforcing the endeavors of the religious supervisors. Therefore, there should be a continuous pressure from the consumer side on the Kosher and Halal marketing system to implement trademarked symbols representing an organization that would be accountable to the consumer which in turn, will assure both the companies and the consumers regarding the authenticity status of the Kosher and/or Halal products being marketed.

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## ORCID

M. A. Motalib Hossain http://orcid.org/0000-0003-2590-5394
Mohd Rafie Johan http://orcid.org/0000-0003-1133-9977

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