

Authentication approach using enzyme-linked immunosorbent assay for detection of porcine substances

R.M.H. Raja Nhari^{1*}, I. Hanish², N.F. Khairil Mokhtar¹, M. Hamid² and A.F. El Sheikha^{3,4,5,6,7*}

¹Laboratory of Halal Science Research, Halal Products Research Institute, Putra Infoport, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia; ²Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia; ³Jiangxi Agricultural University, 1101 Zhimin Road, Nanchang 330045, China; ⁴Bioengineering and Technological Research Centre for Edible and Medicinal Fungi, Jiangxi Agricultural University, 1101 Zhimin Road, Nanchang 330045, China; ⁵Jiangxi Key Laboratory for Conservation and Utilization of Fungal Resources, Jiangxi Agricultural University, 1101 Zhimin Road, Nanchang 330045, China; ⁶Department of Biology, McMaster University, 1280 Main St. West, Hamilton, Ontario, L8S 4K1, Canada; ⁷Department of Food Science and Technology, Faculty of Agriculture, Minufiya University, 32511 Shibin El Kom, Minufiya Government, Egypt; elsheikha_aly@yahoo.com; hafidz@upm.edu.my

Received: 19 August 2018 / Accepted: 6 May 2019

© 2019 Wageningen Academic Publishers

REVIEW ARTICLE

Abstract

Food manufacturers across the world commonly add animal substances in their food products. Some food products may contain porcine substances including pork, gelatine, blood and pepsin. These substances significantly affect the texture, colour or taste of the end products. Aside from enhancing sensory qualities, additional ingredients also contribute to preservation, bulk and nutrition. However, the inclusion of porcine substances might not be suitable among certain communities. One primary concern is fraud labelling which includes hiding the addition of porcine substances in food. Therefore, analytical techniques such as enzyme-linked immunosorbent assay (ELISA) have been developed to detect the porcine proteins in food. The ELISA delivers specificity and sensitivity in detecting the targeted animal species in food. This review provides an overview of the ELISA technique which has been developed for potential detection of porcine substances in laboratory-prepared food samples and commercial food products.

Keywords: ELISA, porcine substances, food authentication, food products

1. Introduction

Swine/domestic pig/hogs (*Sus scrofa domesticus*) provides the most consumed meat worldwide (FAO, 2018). China is the largest country to supply pork followed by the European Union and the United States of America (USDA, 2017). Pork is the meat of the hogs/pigs which is light red in colour prior to cooking. A lard, which is a whitish fat substance, can be found covering the pig's carcass and dispersed throughout the pork. The lard is used in the manufacturing of shaving creams, soaps, cosmetic products, baked goods and other food. Other porcine substances are also used for medical purposes, such as porcine insulin for the regulation of human diabetes and porcine valves as a substitute for human heart valves. Porcine blood is typically

used in meat products while pepsin is commonly added in selected cheese manufacturing. Additionally, porcine gelatine is utilised in the preparation of pharmaceutical and confectionary products. Porcine by-products are also extensively used outside the food industries. For example, porcine skin is used in the production of shoes and clothing, water filters, insulation, rubber, antifreeze, certain plastics, floor waxes, crayons, chalk, adhesives, and fertilizer (US EPA, 2015).

Adulteration of food with porcine products may occur due to cross-contamination or failures to observe proper food handling procedures. More importantly, a common major fraud in the food industry is concealing the presence of porcine substances in food labelling. In these cases,

food manufacturer intent to maximise profit by replacing expensive ingredients such as beef with cheaper porcine alternatives, and conceal it to prevent the loss of market share among certain communities. These communities include Muslims and Jews worldwide who respectively observe Halal and Kosher dietary laws that prohibit the consumption of porcine substances (Regenstein *et al.*, 2003; Rohman and Che Man, 2012). Adulteration of food also harm consumers who are allergic to porcine serum albumin as well as other animal serum albumins from chicken, horse and cow (Asero *et al.*, 1997; El Sheikh *et al.*, 2017; Rupa *et al.*, 2008; Wilson and Platts-Mills, 2018). Based on Ayuso *et al.* (1999), 33 patients (58%) were showed an allergic reaction to raw and cooked pork. Therefore, the authenticity of food products is paramount to consumers and other participants in any food-based economy.

An identification of the target protein or peptide in food requires reliable scientific techniques to determine its animal origin (Murugaiah *et al.*, 2009; Ortea *et al.*, 2016). Food authentication often approaches molecular biology technique such as polymerase chain reaction due to its specificity and sensitivity (El Sheikh, 2015; El Sheikh *et al.*, 2017). However, it requires the level of expertise and costly equipment that may not be accessible to less developed countries. Additionally, the polymerase chain reaction method commonly not able to distinguish DNA between different tissues of the same species (Asensio *et al.*, 2008). Thus, an effective analytical technique for the detection of porcine adulteration in food products is crucially needed to support the law enforcement and consumer protection. One such technique is the application of enzyme-linked immunoassay (ELISA) in food authentication that has been established in earlier years.

This paper critically reviews all studies related to the application of ELISA for the detection of porcine substances in food including pork, gelatine, blood, and pepsin. These studies were analysed based on: type of porcine substances; type of antigen; type of ELISA; type of antibody; limit of detection (LOD) and limit of quantification (LOQ) of the ELISA.

2. Why using ELISA as authenticity tool?

An appropriate labelling law (European Regulation No 1169/2011) obliges the food manufacturers to declare the addition of porcine, intended to help the above-mentioned group of individuals to choose the ideal food for them. The clear labelling of the food products is helpful to protect the individuals who must avoid porcine-containing products (Ofori and Hsieh, 2012). Thus, an effective analytical method for detecting porcine adulteration in food products are crucially needed to support law enforcement and consumer protection.

The ELISA technique has been utilised as an authenticity tool because of its specificity, accuracy, and sensitivity for the detection of antigenic proteins (Asensio *et al.*, 2008; El Sheikh *et al.*, 2017). It is also useful because it is easy to perform, the availability of inexpensive reagents and it allows a large number of sample to be screened or quantitated for the presence of target analyte. For instance, it has been applied for the detection of soy proteins in meat products (Macedo-Silva *et al.*, 2001), detection of peanuts and hazelnuts for health reason of allergic patients (Kiening *et al.*, 2005), identification of fish species for detection of fish adulteration (Fernández *et al.*, 2002) and detection of egg proteins (Yeung *et al.*, 2000).

Despite the numerous advantages of ELISA, this method presents its own limitations. For example, a target antigen that is irregularly denatured during heat processing can lose its epitopes' original forms, which prevents the antigen from being detected by its specific antibody. In addition, the detection of the antigen in the highly processed foods is commonly being inhibited by the ingredients of the food such as lipid, carbohydrates, nucleic acid, salts and other coexistent components. Thus, it will prevent the binding of the antibody against the antigen. The amount of antigen in commercial food is also crucial. The antigen that presents below the limit of detection of ELISA will cause the antibody not being able to detect it. Therefore, there are several solutions have been proposed to overcome the limitations. Firstly, the discoveries of thermostable proteins may eliminate this problem through the development of specific antibodies against them (Jiang *et al.*, 2018; Jones and Patterson, 1985; Kiening *et al.*, 2005; Liu *et al.*, 2006; Macedo-Silva *et al.*, 2001; Mandli *et al.*, 2018). These thermostable proteins are suitable to be used as target antigens by antibodies in the ELISA as they are present in both raw and heated (cooked) conditions of the target samples. Secondly, the pre-treatments of the samples have been conducted to overcome the matrix effect that causes by various components in food (Chiu *et al.*, 2010). However, the limited information about the mechanism of the matrix effect brings trouble to develop reasonable and simple techniques to solve the problem. Figure 1 showed a flow chart for the common ELISA testing to detect porcine from a food sample.

3. What are the ELISA types and what are the differences between them?

There are four types of ELISA including direct, indirect and competitive and sandwich ELISA (Asensio *et al.*, 2008). The direct ELISA involves an antigen coated to a multi-well plate detected by an antibody that has been directly conjugated to an enzyme. The indirect ELISA utilises two antibodies, the capture antibody which is specific to the antigen and the secondary antibody that couples with detection enzyme and bind to the capture antibody to produce a signal

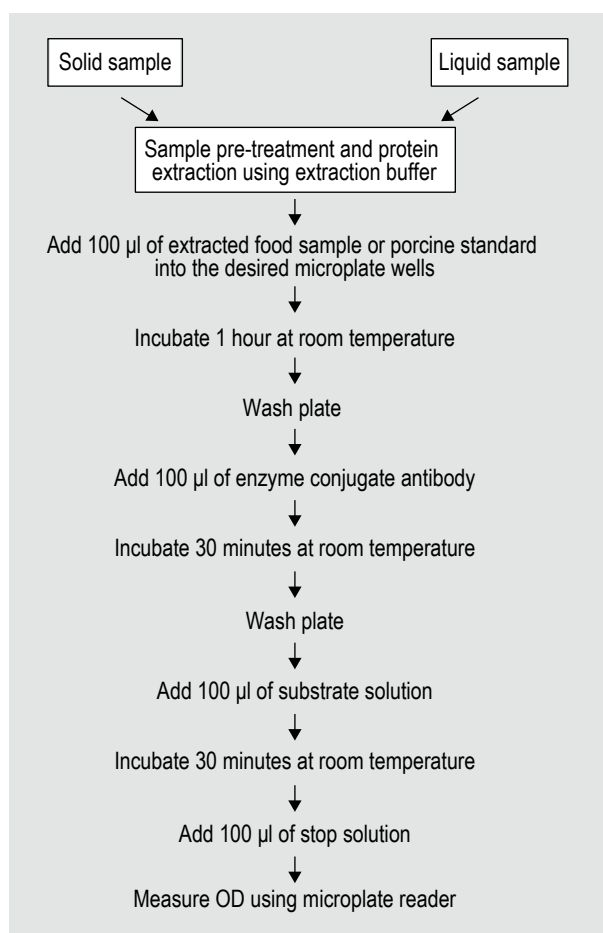


Figure 1. Flow chart for the ELISA testing to detect porcine from a food sample.

from the chromogenic or fluorogenic reaction between the enzyme and substrate. In the competitive ELISA, the captured antibody which is specific to the coating antigen is competitively bound with the mobilised antigen in a sample and the secondary antibody coupled to the detection enzyme bind to the capture antibody (Crowther, 2009). Meanwhile, for the sandwich ELISA, the capture antibody binds to the one epitope of the antigen and the enzyme-conjugated secondary antibody binds to the different epitope of the same antigen. The sandwich ELISA is the most ELISA system used in commercial kits (Schmidt *et al.*, 2012). Whereas, the indirect ELISA is incompatible for repetitive analysis of a large number of samples because of the inconvenient procedures involved especially at the coating step of unknown sample extracts onto the microplates (Crowther, 2009). Table 1 shows advantages versus limitations of the common ELISA types.

4. Type of porcine substances used in food products

Pork

Pork is one of the most consumed livestock meat in the world, which covers over 36% of the world meat intake. It is followed by poultry and beef with about 35 and 22% respectively (Giamalva, 2014). It provides proteins with higher nutritive value and other nutrients than that present in most plant proteins. The pork has been accepted since several years ago in many cultures as the main food source for a variety of processing and cooking methods available.

Table 1. Advantages versus limitations of the common ELISA types (Crowther, 2009; Jantzie *et al.*, 2007; Kragstrup *et al.*, 2013; Schmidt *et al.*, 2012).

Type	Advantages	Limitations
Direct	<ul style="list-style-type: none"> • quick • cross-reactivity of secondary antibody is eliminated 	<ul style="list-style-type: none"> • the primary antibody must be labelled individually • labelling primary antibodies for each specific ELISA system is time-consuming and expensive • no flexibility in the choice of primary antibody label from one experiment to another • minimal signal amplification
Indirect	<ul style="list-style-type: none"> • high sensitivity • flexible • cost-saving 	<ul style="list-style-type: none"> • cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal • an extra incubation step is required in the procedure
Sandwich	<ul style="list-style-type: none"> • high specificity • suitable for complex (or crude/impure) samples • flexibility and sensitivity 	<ul style="list-style-type: none"> • often need validation because of the risk of false positive results
Competitive	<ul style="list-style-type: none"> • high sensitivity • suitable for complex samples • flexibility and sensitivity 	<ul style="list-style-type: none"> • difficulties to find another protein that be recognised by primary antibody

The food labelling regulations always face the issue of adulteration especially the replacement of high-quality meat with the cheaper one in meat products. Undeclared species of the meat products may execute health risks to individuals with allergies to pork and unease certain communities. The case of adding undeclared species seems to be higher in cooked meat products than in raw products which commonly occurs in the market. The pork is one of the potential species to adulterate expensive meat such as beef, goat and venison due to its inexpensive price and its similarity in colour and texture with other meats such as chicken (Bhat *et al.*, 2015). Table 2 shows the substances containing pork and alternative of pork that can be replaced.

Porcine gelatine

The unique properties of animal gelatine that suit in various food products cause a great demand by food manufacturers around the world. Basically, it is a polypeptide obtained by partial hydrolysis of animal collagens by acidic or alkaline treatments. The manufacturing process of gelatine involves

several steps (El Sheikha *et al.*, 2017). The industrial-scale gelatine is produced from the slaughter by-products such as skins and bones which can be obtained at a cheaper price in an acceptable amount. The porcine skin gelatine is a type A gelatine. Generally, the acidic solutions only treated to the tissue of younger porcine that have less covalent bond within collagen as compare to the older porcine so that a good yield and quality of gelatine can be obtained (Abdullah *et al.*, 2016). This food hydrocolloid can be used as a gelling agent, foaming agent, stabiliser, an emulsifier and a foam stabiliser in food products. In meat products, the porcine gelatine can stabilise emulsion in meat and sausages; act as a binder in broths and canned meats (Nur Hanani, 2016). In pharmaceutical products, it is used in the making of soft and hard gel capsules and in dental, it is used as a gel-forming component. It is also applied in cosmetic and medical products. In non-food products, the gelatine is also used in the production of ink jet printing for photography purpose (Hidaka and Liu, 2003; Venien and Leveux, 2005). Hence, determination of the origin of gelatine is therefore of utmost importance for the detection of its properties

Table 2. Substances with porcine and alternative of porcine that can replace porcine (Boudjellab *et al.*, 1998; Giamalva, 2014; Ofori and Hsieh, 2012; Raja Nhari *et al.*, 2012).

Substances containing porcine	Source	Porcine substitutes
Sausages Bacon Salami Hot dog Ham	pork	chicken, turkey bacon, facon, macon, tofu, tempeh
Jellies and fruit gummies Ice creams Marshmallows Emulsifier and foam stabiliser in caramels, yogurt Foamed milk dessert Jellied milk dessert Emulsion stabiliser in meat and sausages Binding agent in broths and canned meats, Pharmaceuticals (soft and hard capsules Gel-forming component in dental pharmaceuticals Thickener in liquid dosage forms, tablets, ointments for mucosal membranes of the mouth, vitamin coating, pastilles, globules) Photography (ink jet printing) Cosmetic and medical products (blood plasma substitutes, gelatine sponges)	porcine gelatine	bovine gelatine, fish gelatine, agar, deacytelated gellan, thermoreversible starch
Dietary supplement Natural meat colorant Binder in meat products Blood sausage Plasma proteins as foaming agent to substitute for eggs; as a gelling and solubilising agent in bakery and yogurt products; and as an emulsifier in cakes and pastries. Surimi and surimi-based foods.	porcine blood	mustard, egg, protein hydrolysate
Cheese	porcine pepsin	bovine rennet, microbial rennet, chemical rennet

and uses (Mohamad *et al.*, 2013, 2016). Table 2 shows the substances containing porcine gelatine and its substitutes.

Porcine blood

The blood from porcine and cattle rather than the blood from other animals is commonly being utilised in food products as they can easily be obtained in large quantities from meat industries as slaughtered-by products. Porcine plasma, the main component of the porcine blood, can provide multiple functional properties including an emulsifier, foaming, gelling and solubilising agent in bakery, cakes, pastries and yogurt products. For the prevention of proteolysis of fish meat by endogenous enzymes, the spray-dried porcine plasma added into the fish and meat-based products could be used as a proteinase inhibitor in order to avoid gel weakening of surimi and surimi-based foods (Benjakul *et al.*, 2004). Table 2 shows the substances containing porcine blood and its substitutes.

Porcine pepsin

The most popular proteases in cheese production are chymosin (from calf rennet or recombinant) and microbial rennet. Other proteases such as pepsin or plant extracts have been used for some specific kind of cheeses but their use is limited due to lower yields of cheese, bitter flavours and texture defect (Rolet-Répécaud *et al.*, 2015). The porcine pepsin, a proteinase found in the porcine stomach act as a coagulating enzyme in Cheddar and other cheeses with a short ripening period. It has been introduced in Europe and elsewhere since the 1960s (Guinee and Wilkinson, 1992). In the United States, the cheese manufacturers consume the mixture of porcine pepsin and bovine or microbial rennet. These enzymes were varied in their specificity, which affects the final quality, taste, rate and type of proteolysis which determine the development of texture and flavour of the cheeses (Rolet-Répécaud *et al.*, 2017). Table 2 shows the substances containing porcine pepsin and its substitutes.

5. Application of ELISA in detection of porcine substances in food

ELISA-based detection of pork in food

The previous study had applied a sandwich ELISA specific to porcine serum albumin for detection of 1-3% (w/w) pork in beef and manufactured beef products made with rusk and other additives (Jones and Patterson, 1985). A sandwich ELISA that used polyclonal antibodies (pAbs) against porcine sarcoplasmic extracts that used for detection of 1-50% (w/w) pork in raw beef (Martín *et al.*, 1988). However, their ELISA was not suitable for the detection of pork in processed beef.

After several years, there were several studies aimed to detect thermostable proteins in pork in order to detect it after the cooking process, which commonly suitable for detection in commercial meat products. A monoclonal antibody (mAb) specific for muscle proteins was used in ELISA for detection of pork in a mixture of 1-100% (w/w) raw beef and chicken meat (Morales *et al.*, 1994). Four mAbs specific to thermal-stable muscle proteins (TSMPs) (mAbs 5H9, 5H8, 2F2, and 8A4) have been developed (Chen *et al.*, 1998). They were reacted with three protein bands (20.5, 22 and 24 kD) from raw pork extract and 24 kD protein band identified as porcine-specific TSMP (troponin I) which also present in cooked pork. The mAb-based ELISA enabled detection of 10 g/kg [1% (w/w)] of pork in raw and cooked meat mixtures.

The ELISA using mAb 5H9 to the porcine TSMP was optimised for detection of pork in cooked meat products (Chen and Hsieh, 2000). The assay specifically detects porcine skeletal muscle except for cardiac muscle, smooth muscle, blood and non-muscle organs. No cross-reactivity was observed with common food proteins. The validity of the assay was evaluated with laboratory formulated and commercial meat samples. The LOD of ELISA was determined as 0.5% (w/w) pork in meat mixtures.

The mAb 5H9 (detection antibody) was paired with the mAb 8F10 (capture antibody) which specific to the porcine troponin I in a sandwich ELISA that capable of detecting low levels of pork in both heat-treated (132 °C for 2 h) and untreated products (Liu *et al.*, 2006). Heat treatment of meat samples did not affect the assay performance. The ELISA was successfully detected 0.05% (w/w) and 0.1% (w/w) of laboratory-adulterated pork in chicken and beef mixtures, respectively. All the mAb-based ELISA which have been developed could be used as reliable methods to detect the trace amounts of pork in meat products to ensure the authenticity of the meat-based products.

ELISA-based detection of porcine gelatine in food

The pAbs against bovine and porcine gelatine was developed to differentiate raw porcine and bovine gelatines using ELISA (Venien and Leveux, 2005). Some pAbs were highly sensitive to the pretreatment process of gelatines. There were pAbs that more sensitive to acidic-treated porcine gelatine except for alkaline-treated porcine bone gelatine. Other pAbs have broad specificity to different animal gelatine. The effectiveness of this ELISA depends on the type of gelatine process, the type of animal used for gelatine (bovine or porcine) and the part to produce gelatine (bone or skin/hide).

In other studies, the new ELISA method was developed for detection of gelatines in food (Doi *et al.*, 2009). They have developed two sandwich ELISAs that were strongly

reacted for bovine and porcine gelatine. The addition of gelatine in all commercial foods which have been stated to contain gelatine was detected by both ELISAs. The studies were also confirmed that when both ELISA were tested commercial foods, the ELISA produced no false positives and no false negatives, except for cooked meat products. In details, the pAb2-pAb1 ELISA was cross-reacted to cooked squid, while the pAb3-pAb3 ELISA did not cross-reacted to it. Thus, the pAb3-pAb3 ELISA method was suitable for detection of alkaline-based bovine and porcine gelatine in foods because it has no cross-reactivity to cooked squid, and produced no false positives or negatives. However, the false positive result for gelatinised cooked meat caused this ELISA method to have its limitation. The factors affecting the effectiveness of this ELISA method are the process of gelatine production.

Besides that, the gelatine is also being adulterated in the edible bird's nest (EBN) in order to increase its net weight prior to sale. The study has been conducted to determine the ability of ELISA to detect porcine gelatine in EBN (Tukiran *et al.*, 2015). They have developed indirect ELISA for detection of porcine gelatine using pAbs. Three indirect ELISAs were developed using pAbs against specific peptide sequences of porcine collagen (I) $\alpha 2$ chain (antigen for pAb1 and pAb2) and collagen (I) $\alpha 1$ chain (antigen for pAb3), which had LODs of 0.12, 0.10 and 0.11 $\mu\text{g/g}$, respectively. However, all pAbs showed cross-reactivity to bovine and fish gelatines; where the pAb1 had slight cross-reactivity to EBNs of orange (optical density=0.2>0.15), cave (0.25>0.15) and house nests (optical density=0.16>0.15). The coefficients of variation was less than 20% shows good repeatability of the ELISA while LOD of the ELISA was ≥ 0.5 ng/ μg (0.05% w/w) porcine gelatine in spiked samples. All pAbs were not suitable for detection of porcine gelatine in EBNs due to cross-reactivity to bovine and fish gelatines even though that the pAb2 has lowest LOD and no cross-reactivity to other EBNs.

The competitive indirect ELISA has also been developed for detection of porcine gelatine in EBNs (Tukiran *et al.*, 2016a). Three competitive indirect ELISAs were developed using their previous developed pAb1, pAb2, and pAb3. The LOD of each ELISAs was 0.033, 0.082 and 0.052 mg/ml, respectively. The IC_{50} of pAb1, pAb2, and pAb3 was 0.265, 0.394 and 0.228 mg/ml, respectively, and the ELISAs were able to recognise porcine, bovine and fish gelatines. The recovery was at the range of 62.8-125.4% for the EBNs spiked with porcine gelatine with coefficients of variation of 2.9-5.4% and 4.7-9.6%, respectively, shows the repeatability of the assays when using pAb3. However, the pAb3 still not suitable to be used as all the pAbs were cross reacted to other animal gelatines.

The gelatine is also being added in confectionery products due to its ability to form soft gels and melts at around body

temperature. The pAbs were developed against porcine specific peptide collagen $\alpha 2$ (I) chain to determine the gelatine's origin in confectionery products using ELISA (Tukiran *et al.*, 2016b). Collagen $\alpha 2$ (I) chain protein was detectable in certain commercial products when analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The ELISA was slight cross-reacted to fish (9.2%) and chicken (6.3%) gelatine. The IC_{50} value was 0.39 $\mu\text{g/ml}$ and the LOD (IC_{10}) was 0.05 $\mu\text{g/ml}$. However, this ELISA was not suitable to detect porcine gelatine due to its cross-reactivity to fish and chicken gelatine that would give false positive result.

ELISA-based detection of porcine blood substances in food

Raja Nhari *et al.* (2016) have developed mAbs against autoclaved porcine blood which potentially being useful for the detection of porcine blood in cooked food using indirect ELISA. While this ELISA may be suitable for detecting porcine plasma in cooked food, its sensitivity and validation have yet to be determined. Meanwhile, the sandwich ELISA using mAbs 19C5-E10 and 16F9-C11 was developed and shown to detect porcine blood specifically (Ofori and Hsieh, 2016). This sandwich ELISA has a capability in detecting $\leq 0.03\%$ (v/v) of porcine blood in cooked (100 °C for 15 min) ground meats or fish. Further studies by Raja Nhari *et al.* (2018) involved the detection of porcine plasma using their previous developed mAb B4E1 in the indirect ELISA. It had a CV less than 20% (good repeatability) and did not cross-react with other meat and non-meat proteins. The sensitivity of the assay is 200 ng/ml of standard porcine plasma solution and 0.25% (w/w) of porcine plasma in spiked raw and cooked fish surimi. The assay did not produce a false positive result for commercial fish surimi that has been labelled free of porcine plasma. Determination of a 60-kD antigenic protein of porcine blood using Western blot confirmed its presence in the plasma fraction.

Another study was conducted using mAb 24C12-E7 in the indirect ELISA that could detect $\leq 1\%$ (v/v) of porcine haemoglobin-containing ingredients in raw ground meats (Ofori and Hsieh, 2017). The LOD for porcine blood proteins in cooked spiked chicken was $\leq 0.5\%$ (v/v) and the LOD for cooked spiked beef was 3 to 10 times higher and even higher (≥ 30 times) for cooked spiked pork. Their mAb-based indirect ELISA is potentially useful for monitoring the presence of spray-dried porcine haemoglobin-containing ingredients in food. Recently, Jiang *et al.* (2018) have highlighted the need for detecting unlabelled of porcine blood in foods. They have developed a mAb13F7-based indirect competitive for the quantification of porcine haemoglobin (PHb) in meat products. This assay had a wide working range from 0.5 to 1000 mg/kg. The LOD and LOQ of the assay is 0.5 and 1000 mg/kg, respectively

and the assay is also precise and reproducible with low coefficient of variances (<20%).

ELISA-based detection of porcine pepsin in food

The ELISA also was developed against porcine pepsin (El-Batawy *et al.*, 1993). Within the range of 1 to 1000 µg/ml of standard porcine pepsin, the ELISA was able to produce a positive reading. The specificity of the antibody was determined using competitive indirect ELISA. The rennet from bovine and *Mucor miehei* was slightly cross-reacted at a higher concentration than 1000 µg/ml (124 and 6-9% inhibition, respectively). The studies were suggested that the ELISA that they have developed could be applied for analysis of the dairy food industry as the method was quick and specific but the cross-reaction with other animal and microbial rennets may give the false positive result.

The ELISA was also developed for the detection of porcine pepsin in rennet mixtures. The lowest concentration of standard porcine pepsin was detected by ELISA is 1 µg/ml. The assay was not cross-reacted to individual or mixtures of bovine rennet and *M. miehei* rennet. The ELISA was used to detect ≥1% (10 µg/ml) porcine pepsin in the bovine and *M. miehei* rennet mixtures. The sensitivity of the ELISA was not affected for samples in a pH range of 5.0 to 7.0. Pre-incubation of porcine pepsin in the presence of ≤10% NaCl was also not affected the sensitivity of the ELISA. No cross-reaction occurred to the milk proteins, and no significant inhibition also occurred with the increasing concentration of casein up to 75 mg/ml.

In 1998, the studies related to the detection of porcine pepsin in a laboratory-prepared soft cheese was performed by using antiserum in competitive indirect ELISA (Boudjellab *et al.*, 1998). The LOD of the ELISA was ~10 ng/ml. The IC₅₀ of the ELISA to detect native and denatured porcine pepsin was ~81 ng/ml. Their ELISA was not cross-reacted to chymosin and proteinase from fungus but cross-reacted to bovine pepsin which showed the limitation of this ELISA. In the laboratory-prepared soft cheese, the ELISA was able to detect porcine pepsin in extracted whey. Furthermore, low amounts of porcine pepsin in curd sample at weak acidic condition was detectable by this ELISA. By using this ELISA, no extraction of porcine pepsin from cheese samples was needed.

6. Conclusions, challenges and future prospects

Porcine substances can be detected in food using ELISA with certain limitations. Most of the previous studies were focused on the detection of pork, porcine gelatine, porcine blood and porcine pepsin using in-house ELISA methods. Its application in previous studies has successfully overcome many of the issues pertaining to porcine adulteration in

food. So far, the most reliable ELISA methods to detect porcine substances were involving the detection of pork and porcine blood due to the ability of their antibodies to differentiate pork from other animal meats and non-meat proteins; and porcine blood from other animal blood and non-blood proteins. However, some of these techniques present several limitations. This includes the inability to detect their targets in processed food, especially in heat-denatured condition. Additionally, the pAbs also have unintended cross-reactivity with proteins from other food or animals. Furthermore, most of the studies did not determine their LOQ of ELISA. Multiple contemporary challenges must be faced by researchers in ELISA-based food authenticity: limited availability of commercial antibodies and kits; problems in the analysis of highly processed food, complex food matrices or genetically modified food; and unsatisfying parallel identification and reliable quantification of multiple biomarkers. The reliability of ELISA depends on the specificity of antibodies to the antigen, cross-reactivity of antibodies to the other proteins, the stability of the antigen in processed food, and sensitivity towards complex food matrix.

Based on this review, new commercial ELISA-porcine detection kits can be developed to detect other porcine proteins in food products that may not being studied yet. So far, the available commercial ELISA kits for detection of porcine proteins in the markets are including ELISA kits for porcine pepsin (12 brands), pork (1 brand), porcine blood (5 brands), and 1 porcine gelatine (1 brand).

As prospects for the near future, the ELISA could be performed together with other sensitive and specific techniques such as DNA-based analyses. This could provide actionable scientific information for the regulatory bodies to protect consumers against fraudulent practices in the food industry. Studies involving the production of monoclonal antibodies that are specific to thermostable porcine proteins must be geared towards ensuring the reliability of ELISA in detecting the target proteins in highly processed food.

Acknowledgements

This work was financially supported by the Universiti Putra Malaysia [GP/2018/9625900].

References

- Abdullah, M.S.P., Noordin, M.I., Ismail, S.I.M., Nyamathulla, S., Jasamai, M., Wai, L.K., Mustapha, N.M. and Shamsuddin, A.F., 2016. Physicochemical evaluation and spectroscopic characterisation of gelatine from shank and toes of *Gallus gallus domesticus*. *Sains Malaysiana* 45: 435-449.
- Asensio, L., González, I., García, T. and Martín, R., 2008. Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). *Food Control* 19: 1-8.

- Asero, R., Mistrello, G. and Falagiani, P., 1997. Oral allergy syndrome from pork. *Allergy* 53: 684-685.
- Ayuso, R., Lehrer, S.B., Tanaka, L., Ibañez, M.D., Pascual, C., Burks, A.W., Sussman, G.L., Goldberg, B., Lopez, M. and Reese, G., 1999. IgE antibody response to vertebrate meat proteins including tropomyosin. *Annals of Allergy, Asthma and Immunology* 83: 399-405.
- Benjakul, S., Visessanguan, W. and Chantarasuwan, C., 2004. Effect of porcine plasma protein and setting on gel properties of surimi produced from fish caught in Thailand. *LWT – Food Science and Technology* 37: 177-185.
- Bhat, M.M., Jalal, H., Para, P.A., Bukhari, S.A., Ganguly, S., Bhat, A.A., Wakchaure, R. and Qadri, K., 2015. Fraudulent adulteration / substitution of meat: a review. *International Journal of Recent Research and Applied Studies* 2: 22-33.
- Boudjellab, N., Grosclaude, J., Zhao, X. and Collin, J.C., 1998. Development of an inhibition enzyme-linked immunosorbent assay for the detection of residual porcine pepsin in a soft cheese sample. *Journal of Agricultural and Food Chemistry* 46: 4030-4033.
- Chen, F.C. and Hsieh, Y.H.P., 2000. Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. *Journal of AOAC International* 83: 79-85.
- Chen, F.C., Hsieh, Y.H.P. and Bridgman, R.C., 1998. Monoclonal antibodies to porcine thermal-stable muscle protein for detection of pork in raw and cooked meats. *Journal of Food Science* 63: 201-205.
- Chiu, M.L., Lawi, W., Snyder, S.T., Wong, P.K., Liao, J.C. and Gau, V., 2010. Matrix effects-a challenge toward automation of molecular analysis. *Journal of the Association for Laboratory Automation* 15: 233-242.
- Crowther, J.R., 2009. The ELISA guidebook, 2nd edition. Humana Press, New York, NY, USA, 566 pp.
- Doi, H., Watanabe, E., Shibata, H. and Tanabe, S., 2009. A reliable enzyme-linked immunosorbent assay for the determination of bovine and porcine gelatin in processed foods. *Journal of Agricultural and Food Chemistry* 57: 1721-1726.
- El Sheikh, A.F., 2015. New strategies for tracing foodstuffs: biological barcodes utilizing PCR-DGGE. *Advances in Food Technology and Nutrition Sciences* 1: 1-7.
- El Sheikh, A.F., Mokhtar, N.F.K., Amie, C., Lamasudin, D.U., Isa, N.M. and Mustafa, S., 2017. Authentication technologies using DNA-based approaches for meats and halal meats determination. *Food Biotechnology* 31: 281-315.
- El-Batawy, M., Zahran, A.S., Madkor, S.A., Smith, C.J., 1993. Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of porcine pepsin as a milk clotting enzyme. *Food and Agricultural Immunology* 5: 57-60.
- European Commission, 2011. European Commission Regulation No. 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. *Official Journal of the European Union* L 304: 18-63. Available at: <http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R1169&from=EN>
- Fernández, A., García, T., Asensio, L., Rodríguez, M.A., González, I., Lobo, E., Hernández, P.E. and Martín, R., 2002. Identification of the clam species *Ruditapes decussatus* (grooved carpet shell), *Venerupis rombooides* (yellow carpet shell) and *Venerupis pullastra* (pullet carpet shell) by ELISA. *Food and Agricultural Immunology* 14: 65-71.
- Food and Agriculture Organization of the United Nations (FAO), 2018. Sources of meat. Available at: http://www.fao.org/ag/againfo/themes/en/meat/backgr_sources.html
- Giamalva, J., 2014. Pork and swine – industry and trade summary. United States International Trade Commission, Washington, DC, USA, pp. 1-84. Available at: https://www.usitc.gov/publications/332/pork_and_swine_summary_its_11.pdf
- Guinee, T.P. and Wilkinson, M.G., 1992. Rennet coagulation and coagulants in cheese manufacture. *International Journal of Dairy Technology* 45: 94-104.
- Hidaka, S. and Liu, S.Y., 2003. Effects of gelatins on calcium phosphate precipitation: a possible application for distinguishing bovine bone gelatin from porcine skin gelatin. *Journal of Food Composition and Analysis* 16: 477-483.
- Jantzie, L.L., Tanay, V.A.M.I. and Todd, K.G., 2007. Methods in immunochemistry. In: Lajtha, A., Baker, G., Dunn, S. and Holt, A. (eds.) *Handbook of neurochemistry and molecular neurobiology: practical neurochemistry methods*, 3rd edition. Springer-Verlag Berlin, Heidelberg, Germany, pp. 193-218.
- Jiang, X., Fuller, D., Hsieh, Y.H.P. and Rao, Q., 2018. Monoclonal antibody-based ELISA for the quantification of porcine hemoglobin in meat products. *Food Chemistry* 250: 170-179.
- Jones, S.J. and Patterson, R.L.S., 1985. Double-antibody ELISA for detection of trace amounts of pig meat in raw meat mixtures. *Meat Science* 15: 1-13.
- Kiening, M., Niessner, R., Drs, E., Baumgartner, S., Krska, R., Bremer, M., Tomkies, V., Reece, P., Danks, C., Immer, U. and Weller, M.G., 2005. Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *Journal of Agricultural and Food Chemistry* 53: 3321-3327.
- Kragstrup, T.W., Vorup-Jensen, T., Deleuran, B. and Hvid, M., 2013. A simple set of validation steps identifies and removes false results in a sandwich enzyme-linked immunosorbent assay caused by anti-animal IgG antibodies in plasma from arthritis patients. *SpringerPlus* 2: 263.

- Liu, L.H., Chen, F.C., Dorsey, J.L. and Hsieh, Y.H.P., 2006. Sensitive monoclonal antibody-based sandwich ELISA for the detection of porcine skeletal muscle in meat and feed products. *Journal of Food Science* 71: M1-M6.
- Macedo-Silva, A., Shimokomaki, M., Vaz, A.J., Yamamoto, Y.Y. and Tenuta-Filho, A., 2001. Textured soy protein quantification in commercial hamburger. *Journal of Food Composition and Analysis* 14: 469-478.
- Mandli, J., El Fatimi, I., Seddaoui, N. and Amine, A., 2018. Enzyme immunoassay (ELISA/immunosensor) for a sensitive detection of pork adulteration in meat. *Food Chemistry* 255: 380-389.
- Martín, R., Azcona, J.I., Casas, C., Hernández, P.E. and Sanz, B., 1988. Sandwich ELISA for detection of pig meat in raw beef using antisera to muscle soluble proteins. *Journal of Food Protection* 51: 790-794.
- Mohamad, A.N., El Sheikh, A.F., Mustafa, S. and Mokhtar, N.F.K., 2013. Comparison of gene nature used in real-time PCR for porcine identification and quantification: a review. *Food Research International* 50: 330-338.
- Mohamad, N.A., Mustafa, S., El Sheikh, A.F., Khairil Mokhtar, N.F., Ismail, A. and Ali, M.E., 2016. Modification of gelatine-DNA interaction for optimised DNA extraction from gelatine and gelatine capsule. *Journal of the Science of Food and Agriculture* 96: 2344-2351.
- Morales, P., García, T., González, I., Martín, R., Sanz, B. and Hernández, P.E., 1994. Monoclonal antibody detection of porcine meat. *Journal of Food Protection* 57: 146-149.
- Murugaiah, C., Noor, Z.M., Mastakim, M., Bilung, L.M., Selamat, J. and Radu, S., 2009. Meat species identification and Halal authentication analysis using mitochondrial DNA. *Meat Science* 83: 57-61.
- Nur Hanani, Z.A., 2016. Gelatine. In: Caballero, B., Finglas, P.M. and Toldrá, F. (eds.) *Encyclopedia of food and health*, 1st edition, Vol. 2. Academic Press, Oxford, UK, pp. 191-195.
- Ofori, J. and Hsieh, Y.H.P., 2017. Immunodetection of porcine red blood cell containing food ingredients using a porcine-hemoglobin-specific monoclonal antibody. *Foods* 6: 101.
- Ofori, J.A. and Hsieh, Y.H.P., 2012. The use of blood and derived products as food additives. In: El-Samragy, Y. (ed.) *Food additive*. InTech, Rijeka, Croatia, pp. 229-256.
- Ofori, J.A. and Hsieh, Y.H.P., 2016. Monoclonal antibodies as probes for the detection of porcine blood-derived food ingredients. *Journal of Agricultural and Food Chemistry* 64: 3705-3711.
- Ortea, I., O'Connor, G. and Maquet, A., 2016. Review on proteomics for food authentication. *Journal of Proteomics* 147: 212-225.
- Raja Nhari, R.M.H., Hamid, M., Rasli, N.M., Omar, A.R., El Sheikh, A.F. and Mustafa, S., 2016. Monoclonal antibodies specific to heat-treated porcine blood. *Journal of the Science of Food and Agriculture* 96: 2524-2531.
- Raja Nhari, R.M.H., Ismail, A. and Che Man, Y.B., 2012. Analytical methods for gelatin differentiation from bovine and porcine origins and food products. *Journal of Food Science* 71: R41-R46.
- Raja Nhari, R.M.H., Khairil Mokhtar, N.F., Hanish, I., Hamid, M., Mohamed Rashidi, M.A. A. and Shahidan, N.M., 2018. Monoclonal antibody-based enzyme immunoassay for detection of porcine plasma in fish surimi. *Food Additives and Contaminants: Part A* 35(5): 807-817.
- Regenstein, J.M., Chaudry, M.M. and Regenstein, C.E., 2003. The kosher and halal food laws. *Comprehensive Reviews in Food Science and Food Safety* 2: 111-127.
- Rohman, A. and Che Man, Y.B., 2012. Analysis of pig derivatives for Halal authentication studies. *Food Reviews International* 28: 97-112.
- Rolet-Répécaud, O., Arnould, C., Dupont, D., Gavoye, S., Beuvier, E. and Achilleos, C., 2015. Development and evaluation of a monoclonal antibody-based inhibition ELISA for the quantification of chymosin in solution. *Journal of Agricultural and Food Chemistry* 63: 4799-4804.
- Rolet-Répécaud, O., Arnould, C., Dupont, D., Gavoye, S., Beuvier, E. and Achilleos, C., 2017. Quantification of pepsin in rennet using a monoclonal antibody-based inhibition ELISA. *LWT – Food Science and Technology* 76: 190-196.
- Rupa, P., Hamilton, K., Cirinna, M. and Wilkie, B.N., 2008. Porcine IgE in the context of experimental food allergy: purification and isotype-specific antibodies. *Veterinary Immunology and Immunopathology* 125: 303-314.
- Schmidt, S.D., Mazzella, M.J., Nixon, R.A. and Mathews, P.M., 2012. Aβ measurement by enzyme-linked immunosorbent assay. In: Sigurdsson, E., Calero, M. and Gasset, M. (eds.) *Amyloid proteins: methods in molecular biology (methods and protocols)*. Vol. 849. Humana Press, New York, NY, USA, pp. 507-527.
- Tukiran, N.A., Ismail, A., Mustafa, S. and Hamid, M., 2015. Enzyme immunoassay for the detection of porcine gelatine in edible bird's nests. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment* 32: 1023-1028.
- Tukiran, N.A., Ismail, A., Mustafa, S. and Hamid, M., 2016a. Determination of porcine gelatin in edible bird's nest by competitive indirect ELISA based on anti-peptide polyclonal antibody. *Food Control* 59: 561-566.
- Tukiran, N.A., Ismail, A., Mustafa, S. and Hamid, M., 2016b. Development of anti-peptide enzyme-linked immunosorbent assay for determination of gelatin in confectionery products. *International Journal of Food Science & Technology* 51: 54-60.
- United States Department of Agriculture (USDA), 2017. Livestock and poultry: world markets and trade. USDA Foreign Agricultural Service. Available at: https://www.fas.usda.gov/psdonline/circulars/livestock_poultry.pdf
- United States Environmental Protection Agency (US EPA), 2015. Agriculture: pork production. National Agriculture Compliance Assistance Center. Available at: https://www.epa.gov/sites/production/files/2015-07/documents/ag_101_agriculture_us_epa_0.pdf
- Venien, A. and Levieux, D., 2005. Differentiation of gelatins using polyclonal antibodies raised against tyrosylated bovine and porcine gelatins. *Journal of Immunoassay and Immunochemistry* 26: 215-229.
- Wilson, J.M. and Platts-Mills, T.A.E., 2018. Meat allergy and allergens. *Molecular Immunology* 100: 107-112.
- Yeung, J.M., Newsome, W.H. and Abbott, M.A., 2000. Determination of egg proteins in food products by enzyme immunoassay. *Journal of AOAC International* 83: 139-143.

