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Evaluation of ELISA for Detecting Porcine Content in Halal Compliance

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Abstract

Pork is strictly forbidden for consumption by the Muslim population. According to the Quran, the consumption is strictly prohibited, even in trace amounts or minimal concentrations. In this study, we aimed to evaluate the sensitivity of ELISA to detect porcine in food. We used six types of pork-containing food samples: raw pork meat, grilled pork skewers, pork oil, pork fat, pork fried rice, and pork meatballs. To ensure sensitivity and reproducibility, each sample was tested in duplicate using undiluted, 10x, and 100x dilutions. Samples were evaluated using spectrometry at an absorbance wavelength of 450 nm. As a result, porcine antigen was detected in raw pork meat, grilled pork skewers, and pork meatballs at OD values > 0.07 (1.012; 1.1266; 0.8166) respectively. In pork meatballs, the presence of porcine antigen at high dilutions was inconsistently observed. Moreover, porcine antigen was not detected in pork oil, pork lard, pork fried rice, or beef soup even in undiluted samples at OD value < 0.07. This study successfully detected the presence of porcine antigens, however, its application is currently limited to meat products. Detection was also less sensitive when applied to processed food. Porcine protein was not detectable in oil and lard samples, nor in processed pork meat products at higher dilutions.

Keywords: *food restriction; halal food; porcine detection; good health and wellbeing; Halal authentication; processed meat detection; ELISA; porcine antigen; food testing*

1. Introduction

Halal refers to practices that adhere to Islamic guidelines as outlined in the Quran and Hadith (Kamali, 2021; Shahdan et al., 2016; Ullah et al., 2022). The term 'Halal' is derived from Arabic, meaning 'permissible,' while its opposite, 'Haram,' means 'forbidden' (Al-Hajla, 2017; Butt et al., 2021; Samori et al., 2014). Halal guidelines apply not only to food but also to pharmaceuticals, cosmetics, textiles, and footwear (Hossain et al., 2021; Khan, & Shaharuddin, 2015). In terms of food and beverages, halal restrictions include prohibitions on pork, carrion, blood, alcoholic drinks, and animals that have not been slaughtered in the name of Allah (Abd El-Hack et al., 2018; Fuseini, 2023). Among these items, pork is explicitly prohibited in the Quran, serving as a means of spiritual and moral purification for Muslims (Buntoro et al., 2023; Rahim et al., 2022; Soraji et al., 2017).

One of the main indicators of pork is its porcine nature, referring to a protein associated with swine or pigs. This protein serves as a common indicator in halal food (Alfred et al., 2019; Cook, & Phuc, 2019). Several kits are available on the market to detect porcine protein. One of the top methods is a Polymerase Chain Reaction (PCR), which checks for the presence of pork DNA. Another is the ELISA (Enzyme-Linked Immunosorbent Assay) method,

which detects porcine antigens (Wang et al., 2021). PCR is preferred chosen when highly sensitive and specific results are needed, while ELISA is valued for its ease of use and relatively quick results.

Developing of a user-friendly halal detection kit is necessary to enable individuals to verify the halal quality of their food, particularly regarding pork content (Foroutan et al., 2019). The *enzyme-linked immunosorbent assay* (ELISA), a widely used immunoassay, provides a straightforward, quick, highly precise means for identifying non-halal pollutants, like swine proteins. The test uses antigen-antibody interactions, in which certain antibodies trap a target molecule such as porcine gelatin, causing a visible enzymatic response. A microplate reader may then be used to measure this colorimetric or fluorescence-based signal, thereby offering a clear indication of contamination (Choosang et al., 2024).

ELISA is relatively simple to perform compared to more sophisticated analytical methods like mass spectrometry or high-performance liquid chromatography (HPLC), which require less sample preparation and specific training (Kritsiriwuthinan et al., 2021). One advantage of ELISA for porcine detection is its reduced testing time compared to more conventional analytical methods. While classic chemical and molecular tests like polymerase chain reaction (PCR) for DNA analysis often require advanced sample preparation and long incubation periods, ELISA can deliver results within a few hours, considerably improving efficiency in food safety monitoring (Smirnova et al., 2020). Therefore, ELISA remains a valuable tool for halal testing, ensuring compliance with Islamic dietary requirements through a balance of sensitivity, speed, and ease of use.

2. Objectives

In this study, we aim to evaluate the sensitivity of ELISA in detecting porcine content in food. This study focuses on assessing ELISA performance as a standalone method, without PCR comparison. Positive and negative controls will be employed to confirm the selective binding of the antibody to porcine proteins. The results of this study will serve as preliminary data for the future development of our rapid diagnostic kit for detecting porcine content in food.

3. Materials and Methods

3.1 Sample Preparation

This study used six different types of pork-containing food samples: raw pork meat, grilled pork skewers, pork oil, pork fat, pork fried rice, and pork meatballs. For solid samples, 0.1 grams of the sample were ground and mixed with 1 mL of diluent buffer. The mixture was shaken vigorously for 30 seconds at room temperature and then centrifuged at 100xg for 3 minutes. The upper layer was used for testing. For liquid samples, the samples were diluted twice in blocking solution (2% Bovine Serum Albumin (BSA) (Sigma #A2153), 0,1% Tween-20 (Sigma #P9416), in Phosphatase Buffer Saline (PBS) (Sigma #P2272) and used directly for the test. The appearance of the prepared samples is shown in Figure 1 and the scheme of the experiment is shown in Figure 2. To ensure sensitivity and reproducibility, each sample was prepared at three concentrations: undiluted, 10-fold diluted, and 100-fold diluted, and tested in duplicate. Three negative controls were used in this experiment: a control for non-porcine content (raw beef meat), a control for antigen-antibody specificity binding (blocking solution), and a control for sterility indicators during lab work (diluent buffer).

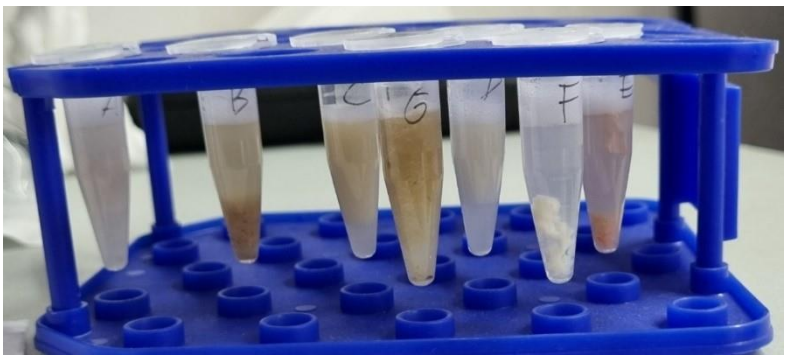

Figure 1 Sample preparation. (A) Raw pork meat, (B) Grilled pork skewers, (C) Pork oil, (D) Pork lard, (E) Pork fried rice, (F) Pork meatball, (G) Beef soup

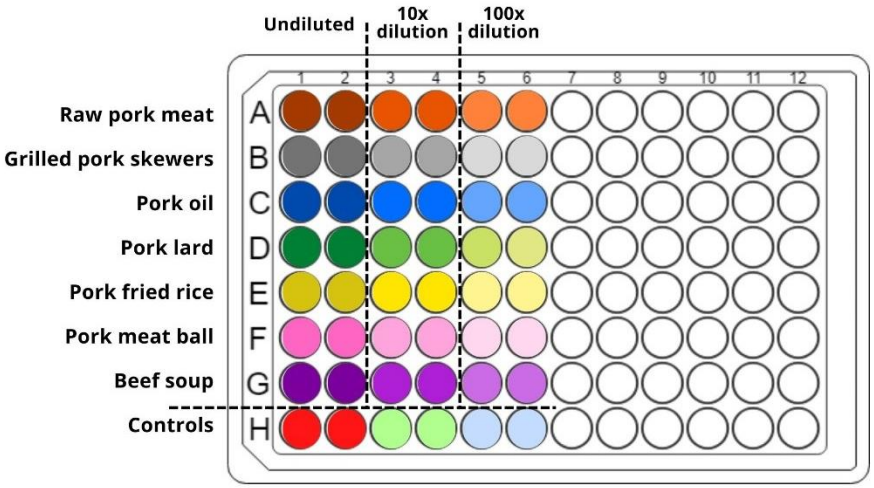

Figure 2 Sample preparation was conducted in a 96-well plate with seven different types of samples, arranged from row A to G. Each sample was prepared in duplicate at three concentrations: undiluted, 10x diluted, and 100x diluted. In row H, the red color indicates the negative porcine control (raw beef meat), the green color indicates the negative control for antibody binding (blocking solution), and the blue color indicates the blank control (diluent buffer).

3.2 Sample Detection Based on ELISA Method

This experiment was conducted using a 96-well plate (Sigma #CLS3508). A volume of 100 µL of each sample and control was added to the corresponding wells. Three controls were used in this experiment: a negative control for non-porcine content (raw beef meat), a negative control for antibody binding (blocking solution), and a blank control (diluent buffer). The plate was incubated overnight at 4°C. Following incubation, the plate was washed three times for 2 minutes each using washing buffer (PBS). Each well received 100 µL of the primary antibody (Rabbit anti-Porcine, Sigma #P0916, 1:10.000), and the plate was then incubated for four hours at room temperature. The plate was washed three times following this incubation, then 100 µL of the secondary antibody (Goat anti-Rabbit HRP Conjugate, Invitrogen #31460, 1:10.000) was added. The plate was incubated at room temperature for one hour. The plate was subsequently washed three times, and each well received 100 µL of the 3,3',5,5' tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific #34028). The plate was incubated for 10 minutes at room temperature, avoiding light exposure. Finally, 100 µL of stop solution (Invitrogen #SS03) was added to each well, and the absorbance was measured within 30 minutes using a spectrometry ELISA reader at a wavelength of 450 nm. Each sample was tested in duplicate.

4. Results

Various samples and controls were prepared and assessed using ELISA. The results were evaluated using spectrometry at an absorbance wavelength of 450 nm, as presented in Figure 3 and Table 1. These optical density (OD) values were interpreted to determine the presence of porcine content, with a cut-off OD value of greater than 0.07 indicating positive results. The cut-off value of 0.07 was determined based on the OD value of the negative control for non-porcine content (raw beef meat). If the optical density (OD) value of a sample exceeded the cut-off, it was considered positive. If the OD value was below the cut-off, it was considered negative. If the OD value was lower than the cut-off, the sample was considered negative.

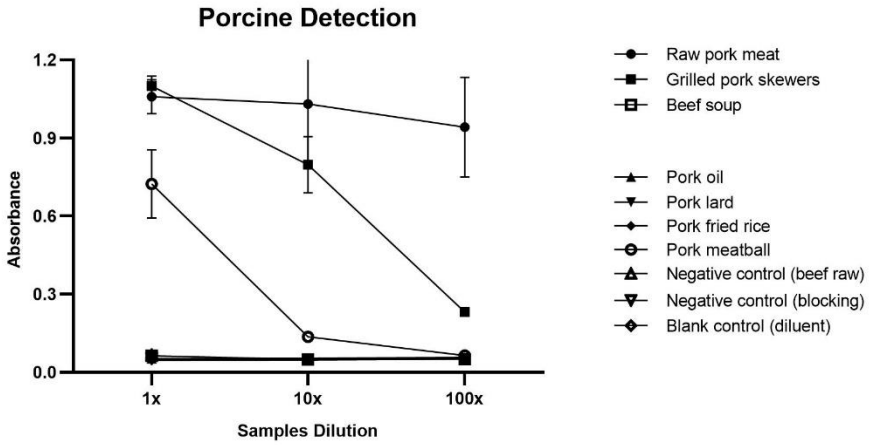

Figure 3 Positive sample is indicated by a high absorbance value, typically exceeding a pre-determined cut-off (> 0.07)

Table 1 Absorbance values of different food samples measured by ELISA at 450 nm

Groups	1x		10x		100x	
	1	2	3	4	5	6
A	1.0125	1.1046	0.8713	1.1893	0.8066	1.0765
B	1.1266	1.0723	0.8744	0.7214	0.2212	0.2427
C	0.0525	0.0522	0.0451	0.0622	0.0671	0.0485
D	0.0494	0.0482	0.0469	0.0522	0.0563	0.0465
E	0.0465	0.0460	0.0457	0.0495	0.0556	0.0478
F	0.8166	0.6307	0.1383	0.1351	0.0763	0.0529
G	0.0663	0.0616	0.0494	0.0497	0.0497	0.0517
H	0.0691	0.0644	0.0588	0.0483	0.0595	0.0493

Noted: The samples are categorized into three conditions: Undiluted (columns 1 and 2), 10x diluted (columns 3 and 4), and 100x diluted (columns 5 and 6). Each row corresponds to a specific sample type: (A) Raw pork meat, (B) Grilled pork skewers, (C) Pork oil, (D) Pork lard, (E) Pork fried rice, (F) Pork meatball, (G) Beef soup. In row H, the red color indicates the negative porcine control (raw beef meat), the green color indicates the negative control for antibody binding (blocking solution), and the blue color indicates the blank control (diluent buffer). Samples exceeding OD 0.07 are considered positive for porcine antigen detection.

Table 2 Qualitative interpretation of ELISA results based on absorbance threshold (OD > 0.07 indicates positive detection of porcine antigen)

Groups	1x		10x		100x	
	1	2	3	4	5	6
A	+	+	+	+	+	+
B	+	+	+	+	+	+
C	-	-	-	-	-	-
D	-	-	-	-	-	-
E	-	-	-	-	-	-
F	+	+	+	+	+	-
G	-	-	-	-	-	-
H	-	-	-	-	-	-

Noted: The cutoff value for a positive result indicative of porcine content is an OD value greater than 0.07. The samples are categorized into three conditions: Undiluted (columns 1 and 2), 10x diluted (columns 3 and 4), and 100x diluted (columns 5 and 6). Each row corresponds to a specific sample type: (A) Raw pork meat, (B) Grilled pork skewers, (C) Pork oil, (D) Pork lard, (E) Pork fried rice, (F) Pork meatball, (G) Beef soup. In row H, the red color indicates the negative porcine control (raw beef meat), the green color indicates the negative control for antibody binding (blocking solution), and the blue color indicates the blank control (diluent buffer).

Table 2 shows that porcine antigen can be detected in both raw pork meat and grilled pork skewers, even at a 100x dilution. Porcine antigen detection in meatball samples at higher dilutions approached the OD cut-off threshold (0.07), with readings fluctuating between positive and negative interpretations, suggesting the assay is near its limit of reliable detection for this sample type. Furthermore, porcine antigen was not detected in pork oil, pork lard, or pork fried rice, even in undiluted samples. This suggests that porcine antigen detection in processed meat is less sensitive compared to raw meat or minimally processed food. This reduced sensitivity could be due to a limited amount of antigen in the sample or the cooking process potentially destroying the antigen's binding ability. As controls, all the three controls showed negative results across all dilution series.

5. Discussion

The results clearly indicate that the current ELISA method is effective for detecting porcine antigens in pork meat but not in processed forms, such as oil or lard. Additionally, the detection capability in grilled meat, fried rice, and meatballs is limited to specific concentrations. These findings highlight significant limitations in the assay's ability to detect porcine content across various food products.

During food processing, meat undergoes procedures that can significantly alter the physical and chemical properties of proteins. For instance, high-temperature cooking methods such as grilling or frying can substantially impact protein structure (Dolch et al., 2019; Kamankesh et al., 2019; Schmid et al., 2022). The incorporation of various ingredients during processing may also induce structural changes in proteins, potentially reducing the concentration of the antigen in the food sample (Jeong et al., 2023; Segura-Gil et al., 2019).

In the case of oil and lard samples, the primary challenge arises from their lipid-rich composition. The high lipid content in oil and lard can interfere with the antibody's ability to bind effectively to porcine antigens. Lipids may form a matrix that reduces the interaction between antigens and antibodies, making it difficult for the antibody to detect the antigen (Watanabe et al., 2021; Yoon et al., 2021). Therefore, for oil and lard samples, it is recommended to involve additional steps such as protein isolation, protein extraction, protein precipitation, or other assay modifications in order to isolate and concentrate the protein from the lipid matrix, as well as to enhance the antibody's binding efficiency in lipid-rich environments (Kim et al., 2023; Yang et al., 2020). In addition, exploring alternative antibodies could be utilized to modify the existing ELISA setup to improve sensitivity (Smirnova et al., 2020).

Food processing techniques such as cooking, frying, or oil extraction can alter or degrade protein structures, thereby reducing the detectability of porcine antigens using protein-based assays like ELISA. In this study, the cut-off optical density (OD) for a positive result was established at 0.07, based on the OD value of the negative control (raw beef meat). This threshold is substantially lower than that of a commercially available (Global Haltech, #HTEP-01-906), which uses a cut-off of 1.3 (Speedy Assay, n.d.). The lower threshold suggests that the method employed in this study may offer enhanced sensitivity, allowing the detection of trace amounts of porcine antigen that might otherwise remain undetected. However, using a lower cut-off value can also increase the risk of false-positive results due to background signal or non-specific binding, highlighting the need for further specificity validation. These findings serve as a foundation for the development of a rapid, field-deployable lateral flow immunoassay for halal verification. Given the limitations of ELISA-based detection alone, future research should incorporate complementary methods such as PCR, which enables highly sensitive and specific detection of porcine DNA, especially in complex or processed food matrices.

6. Conclusion

This study successfully detected the presence of porcine antigens, however its application is currently limited to meat products. Porcine protein was not detectable in oil and lard samples, nor in processed pork meat products at elevated dilutions. The primary challenges include the low concentration of proteins in the samples, issues related to protein solubility and stability, and the lipid-rich environment in oil and lard samples. Further research is needed to enhance the sensitivity of detection and the affinity efficiency of antigen-antibody binding. This may be achieved through approaches such as using alternative antibodies, optimizing protein isolation methods, or employing PCR-based validation. These improvements are essential for ensuring reliable detection of porcine antigens across a broader range of food products.

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