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Nano- and Bio-technological Advancement to assist in the Determination of Halal Products

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ABSTRACT

Aims: Halal industry science can be defined as the experimental investigation of the consumable product by using scientific (analytical) method to reveal its contents, thus assisting to determine the product is halal or not. This scope can be further extended to address any relevant issues on the halal products involving scientific and technological advancements. This definition is contrived for the first time in this article by the author. The present study was conducted in collaboration with university, industry, professional laboratories and governmental organization, which was aimed in finding out how effective the currently available analytical methods especially when the results were targeted to be used in the determination of the Halal products. Furthermore, in this study, the successful development of a protein-based (immuno-chromatography) test kit for the purpose was explained. Methodology and results: DNA extraction was performed using commercially available DNA extraction kit from QIAGEN or NEOGEN. The DNA extraction was performed in duplicate for each sample. PCR-conventional was performed using Thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems). Porcine and Bovine specific primers for mitochondrial DNA sourced from Food and Agricultural Materials Center, Japan (FAMIC) were used. In addition, for comparison NEOGEN primer specific for porcine genomic DNA was used for one sample. A total of 4 commercial products (3 food/snack, 1 functional cosmetic) were tested in this study. Among these, except 1 marshmallow product which might be fish DNA positive, 3 were found as porcine DNA positive. One of the porcine DNA positive product failed to show the same result when it was tested using the commercial kit-NEOGEN- containing porcine genomic DNA. None of these products were found as bovine DNA positive. Conclusion, significance and impact of study: The determination of the Halal is a very sensitive issue. Therefore, in this study, we have concluded that in determination of the Halal in a processed and commercialized product by employing a single approach or method especially when targeting DNA is not enough to confirm the authenticity of the test result due to possible limitation of the method used. We have proven that the primers for mitochondrial DNAs sourced from FAMIC, Japan could be more reliable for the purpose. The effective collaboration between industry, academia and related professional organizations for developing innovative Halal test kit successfully is critical.

***Keywords*:** Biotechnology; Determination of halal products; Halal industry science; Nanotechnology.

INTRODUCTION

As processed and prepared ready-to-eat food products with animal origins have become increasingly available to consumers, due to technological advances, the possibility of fraudulent adulteration and substitution of the expected species (source) with other sources has also increased. The same goes to confectionery and functional cosmetic products especially containing gelatin or collagen peptides. Such practices pose a substantial concern to consumers in terms of economic loss, allergies, religious observance, loss of traceability, and food safety. In many cases, porcine derivatives are used due to cheaper price and readily available. For Muslim consumers, the major authenticity concerns are in meat and meat products include porcine gelatin, collagen, fat, and so on. The analytical methods used for Halal authentication of meat and meat products include: Polymerase Chain Reaction (PCR); Enzyme Linked Immunosorbent Assays (ELISA); Mass spectrometry; Chromatography, Electronic nose and Spectroscopy. An overview of the currently available analytical methods or techniques is given in Table 1. The analytical methods currently in use to detect the presence of porcine materials are mainly protein and DNA-based. These are described below.

DNA-based detection

PCR is capable of amplifying very few copies of DNA and its detection limit is much lower than what is observed with protein based assays. PCR amplification is based on hybridization of specific oligo-nucleotides to a target DNA and synthesis of million copies flanked by these primers. The simplest PCR strategy applied to evaluate presence of any species in a meat product is the amplification of DNA fragments, followed by agarose gel electrophoresis for fragment size verification. To successfully detect a species with PCR, adequate genetic markers are chosen to develop the assay. Either nuclear or mitochondrial genes can be targeted (Fajardo *et al*., 2008). However, the use of mitochondrial DNA (Mt DNA) offers a series of advantages over cell nucleus DNA. Mitochondrial DNA facilitates PCR amplification, even in cases where the availability of DNA template after its ex-traction is insufficient for detection (Murugaiah et *al.*, 2009). This is attributed to the fact that Mt DNA is several fold more abundant than that of nuclear genome; each mitochondrion is estimated to contain 2 to 10 Mt DNA (Murugaiah *et al*., 2009). Furthermore, Mt DNA evolves much faster than nuclear DNA and henceforth contains more sequence diversity facilitating the identification of phylogenetically related species (Fajardo *et al*., 2010; Girish et al., 2005; Murugaiah *et al*., 2009). Among the mitochondrial genes, cytochrome b (cyt b) (Aida et al., 2005; Murugaiah *et al*., 2009) and 12S rRNA (Chen *et al*., 2010; Girish *et al*., 2005) are the most commonly used markers in the development of DNA methods for meat species authentication.

Protein-based detection

Porcine protein, due to it is being cheap and readily available, might fraudulently be used to substitute other animal proteins. ELISA is the most commonly used method to detect animal proteins and a number of commercial immunoassays are available. Chen and Hsieh (2000) were the first ones to develop the immunoassay (ELISA) using a monoclonal antibody to a porcine thermostable muscle protein for detection of pork in cooked meat products. They observed no cross-reactivity with common food proteins. By employing this technology, the first pork detection kit was developed in Japan by a Japanese company (Okamoto, 2016). This kit is immuno-chromatographic employing nano-sized colloidal gold

particles to detect presence of pork in food samples. It can detect pork in both raw and cooked food. It allows rapid detection of pork in food samples at low cost without using any special equipment or requiring skill. The basic principle of immune-chromatographic kit is shown in Figure 1.

Table 1: Summary of analytical techniques applicable in the halal authentication of meat products♦.

Authenticity issue	Analytical Techniques	References
Pork adulteration		Murugaiah *et al*. (2009), Aida, Che Man, Raha, and Son (2007), and Aida *et al*. (2005)
Species identification	PCR-RFLP	
	Real time PCR	Martín *et al*. (2009), Kesmen, Gulluce, Sahin, and Yetim (2009), Tanabe *et al*. (2007), Fumière, Dubois, Baeten, von Holst, and Berben (2006), and López-Andreo, Garrido-Pertierra, and Puyet (2006)
	Species-specific PCR	Soares, Amaral, Mafra, and Oliveira (2010), Alaraidh (2008), Che Man *et al*. (2007) and Montiel-Sosa *et al*. (2000)
	RAPD	Martinez and Malmheden Yman (1998)
	PCR sequencing	Karlsson and Holmlund (2007)
Pork protein	ELISA	Chen and Hsieh (2000); Chen and Hsieh (2000)
	Chromatography	Chou *et al*. (2007)
	Peptide examination	Aristoy and Toldra (2004)
	Isoelectric focusing	Hofmann (1985)
Pork fat (lard)	FTIR spectroscopy	Rohman, Sismindari, Erwanto, and Che Man (2011a, 2011b), Che Man, Abidin, & Rohman, 2010, Rohman and Che Man (2011a, 2011b), Rohman and Che Man (2009), Che Man, Gan, NorAini, Nazimah, and Tan (2005), Che Man, Syahariza, Mirghani, Jinap, and Bakar (2005) and Che Man and Mirghani (2001)
	DSC	Marikkar, Ghazali, Man, and Lai (2003) and Marikkar, Lai, Ghazali, and Che Man (2001)
	Electronic nose	Nurjuliana, Che Man, and Mat Hashim (2011a), Nurjuliana, Che Man, Mat Hashim, and Mohamed (2011b), Che Man, Gan, *et al*. (2005), and Che Man, Syahariza, *et al*. (2005)
Blood plasma	Isoelectronic focusing	Bauer and Stachelberger (1984)
	ELISA	Church and Hart (1995)
	Immunodiffusion	Price, Hart, and Church (1992)
	LC – MS/MS	Grundy *et al*. (2007) and Grundy *et al*. (2008)

♦Source: Nakyinsige et al. (2012).

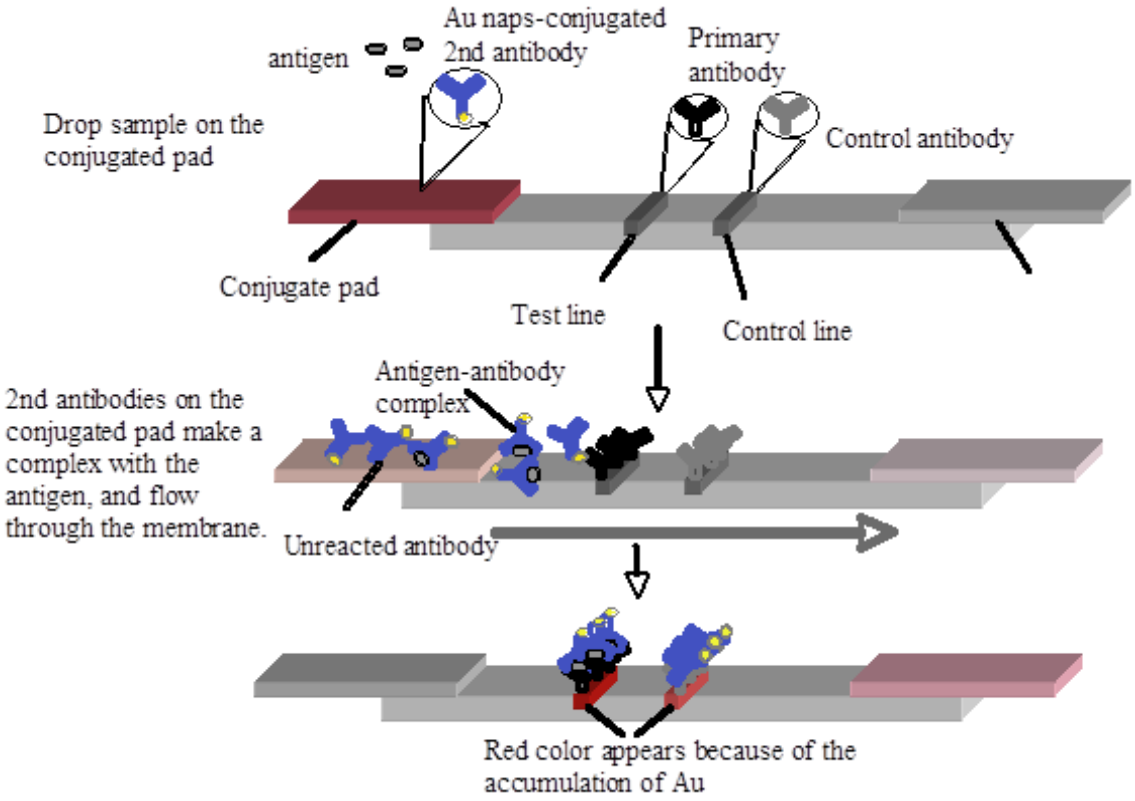

Figure 1 The basic principle of the immuno-chromatographic kit.

MATERIALS AND METHODS

Detection of Porcine and Bovine DNAs from different products containing gelatin/collagen from different sources

DNA extraction was performed using DNA extraction kit from QIAGEN. About 0.25g DNA per sample was extracted. The DNA extraction was performed in duplicate for each sample. The conventional-PCR was performed using Thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems). Porcine and Bovine specific primers for mitochondrial DNA were used. Each primer sequence is shown in Table 2. In addition, in order to check that DNA was extracted successfully, the PCR using the primer for vertebrate detection was also performed. The PCR condition is summarized in Table 3.

Table 2 Sequence of Porcine and Bovine specific primers.

Target	Primer	Sequence
Porcine	Forward	GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA
	Reverse	GCT GAT AGT AGA TTT GTG ATG ACC GTA
Bovine	Forward	GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA
	Reverse	CTA GAA AAG TGT AAG ACC CGT AAT ATA AG

Table 3 Condition of PCR

Step	Temperature (°C)	Time	Cycle
Initial denaturation	94	1 min	1
Denaturation	94	30 sec	
Annealing	60	30 sec	30
Extension	72	30 sec	
Final Extension	72	7 min	1
Hold	4	∞	

Comparative study on the PCR-based detection of the Porcine and Bovine genes using different primers

A. Neogen kit method:

Primer: Neogen Porcine primer-genomic DNA
DNA: 20 ng
PCR steps:

Step	Temperature (°C)	Time	Cycle
Initial denaturation	94	10 min	1
Denaturation	94	15 sec	
Annealing	64	15 sec	30
Extension	72	15 sec	
Final Extension	72	3 min	1
Hold	4	∞	

B. FAMIC method:

Primer:FAMIC Porcine primer-mitochondrial DNA
DNA: 20 ng
PCR steps:

Step	Temperature (°C)	Time	Cycle
Initial denaturation	95	9 min	1
Denaturation	92	30 sec	
Annealing	60	1 min	45
Extension	72	1 min	
Final Extension	72	5 min	1
Hold	4	∞	

Primer:FAMIC Bovine primer-mitochondrial DNA
DNA: 20 ng
PCR steps:

Step	Temperature (°C)	Time	Cycle
Initial denaturation	95	9 min	1
Denaturation	92	30 sec	
Annealing	55	30 sec	45
Extension	72	30 sec	
Final Extension	72	5 min	1
Hold	4	∞	

RESULTS

Detection of Porcine and Bovine DNAs from different products containing gelatin/collagen from different sources

Table 4: Summarized results of the detection of porcine, bovine and vertebrate DNA

	Porcine	Bovine	Vertebrate
Sample 1	Not detected	Not detected	Detected*
Sample 2	Detected*	Not detected	Not detected
Sample 3	Detected*	Not detected	Not detected
Sample 4	Detected*	Not detected	Detected*

*When one of the test samples from duplicate was found positive, it was taken as the positive (as shown in the following three figures).

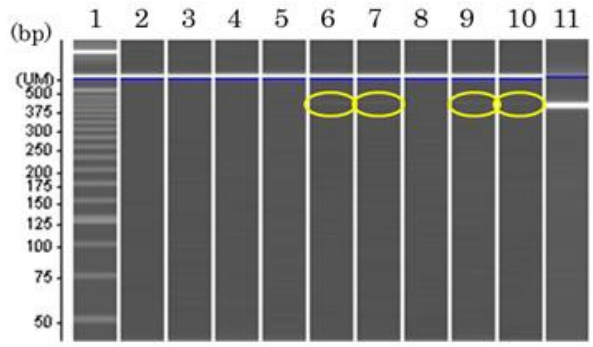

Figure 2 Result of PCR using Porcine specific primer; Lane 1: Marker (25bp ladder), 2: Negative control, 3: sample 1 (n=1), 4: sample 1 (n=2), 5: sample 2 (n=1), 6: sample 2 (n=2), 7: sample 3 (n=1), 8: sample 3 (n=2), 9: sample 4 (n=1), 10: sample 4 (n=2), and 11: Positive control [Microchip electrophoresis system (MultiNA), Shimadzu Corporation].

Assumptions on the detection of porcine, bovine and vertebrate DNA from four commercialized products are listed below:

1. Not a single product showed positive result for bovine material.
2. Three products (one marshmallow, one Jelly, one functional cosmetic) showed positive result for porcine material.
3. Two products (one marshmallow, one functional cosmetic) showed positive result for vertebrate material.

Only one of the two marshmallow products might contain fish material (gelatin) since it was found porcine negative.

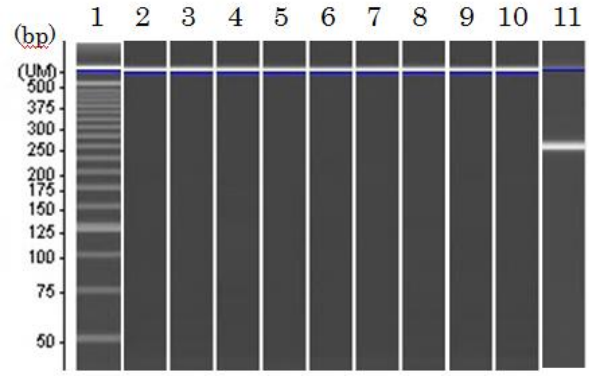

Figure 3 Result of PCR using Bovine specific primer; Lane 1: Marker (25bp ladder), 2: Negative control, 3: sample 1 (n=1), 4: sample 1 (n=2), 5: sample 2 (n=1), 6: sample 2 (n=2), 7: sample 3 (n=1), 8: sample 3 (n=2), 9: sample 4 (n=1), 10: sample 4 (n=2), and 11: Positive control [Microchip electrophoresis system (MultiNA), Shimadzu Corporation].

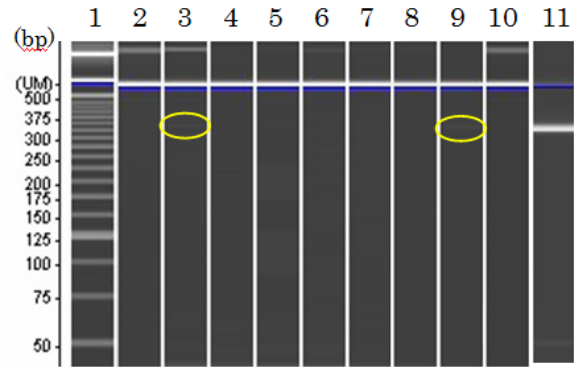

Figure 4 Result of PCR using the primer that common to a vertebrate; Lane 1: Marker (25bp ladder), 2: Negative control, 3: sample 1 (n=1), 4: sample 1 (n=2), 5: sample 2 (n=1), 6: sample 2 (n=2), 7: sample 3 (n=1), 8: sample 3 (n=2), 9: sample 4 (n=1), 10: sample 4 (n=2), and 11: Positive control [Microchip electrophoresis system (MultiNA), Shimadzu Corporation].

Comparative study on the PCR-based detection of the Porcine and Bovine genes using different primers

Result-1 (DNA concentration)

The values are shown in the following table
DNA extraction kit: Neogen (Speciation)
Samples:

No	Sample ID	DNA (ng/µl)	260/280
1	M-A	12.66	1.50
2	M-B	22.44	1.38
3	T-A	10.49	1.63
4	T-B	12.19	1.52

Result-2 (PCR-Electrophoresis)

A. Neogen kit method: As shown in the Figure 5, no porcine DNA was detected in any of the two samples tested in duplicates.

B. FAMIC method: As shown in the Figure 6, Porcine DNA was detected for the two samples though in single from duplicate (M-B and T-A). No bovine DNA was detected from the two samples tested.

Assumptions on the comparative study using different primers are listed below:

1. The porcine DNA was detected in the both samples (marshmallow M-B and T-A) when using only the FAMIC method. These results indicate that both the marshmallows might have a small amount of porcine DNA.
2. The bovine DNA was not detected in any of the two samples tested under the same condition.

DISCUSSION

In this study, we have reported on comparing the specific and qualitative detection methods for porcine and bovine materials in the processed food products (marshmallows

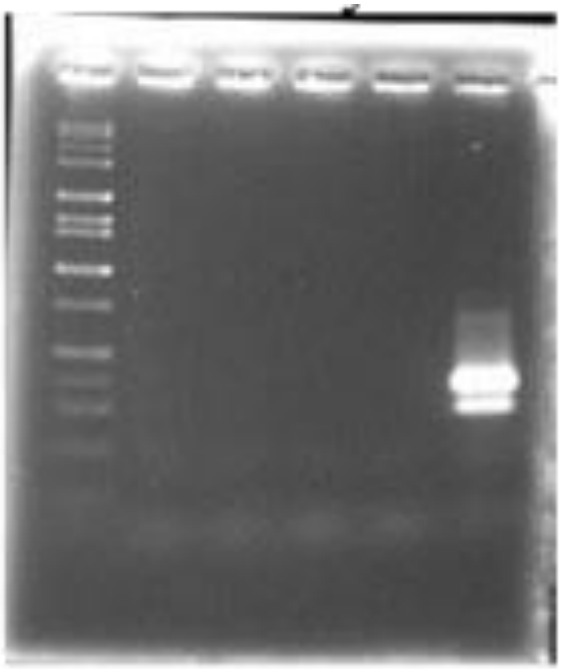

Figure 5 Porcine DNA; PCR product size: 380-bp (housekeeping fragment), 314-bp (porcine fragment); from left: marker, M-A, M-B, T-A, T-B, positive control (380-bp, 314-bp).

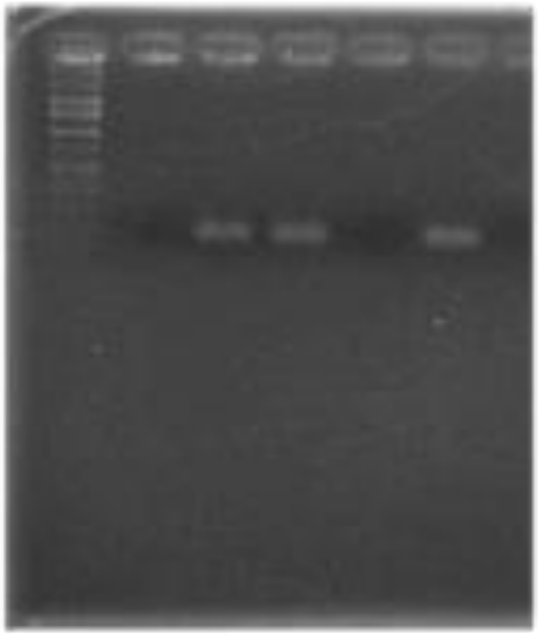

Figure 6 Porcine DNA; PCR product size: 126-bp; from left: M-A, M-B, T-A, T-B, positive control.

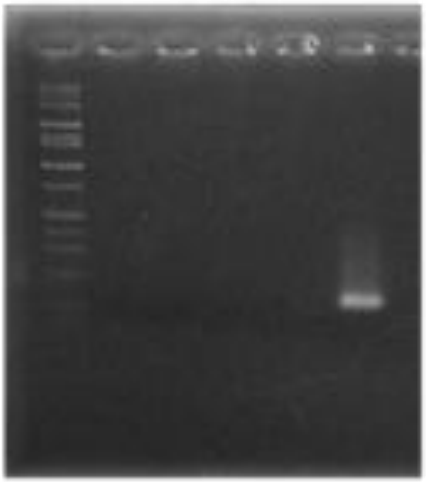

Figure 7 Bovine DNA; PCR product size: 126-bp; from left: M-A, M-B, T-A, T-B, positive control

and jelly containing gelatin) and functional cosmetic product containing collagen peptides by employing conventional PCR and two different primers (nuclear and mitochondrial genes). Earlier, two other groups also reported about the successful application of the conventional PCR in meat species detections including from the processed foods (Tanabe *et. al*., 2007; Matsunaga *et. al*., 1999). These conventional PCR methods are simple and useful. That is why we have employed these. The choice of the target gene and the design of the primers have a great impact on the sensitivity and specificity of a detection system. It is well-known that very sensitive PCR assays can be established when the primer target is a multicopy gene, such as a mitochondrial gene (Holzhauser *et. al*., 2006). In this study, we chose both mitochondrial and nuclear (genomic) genes as the target to detect porcine and bovine materials for comparing the efficiency of these two primers while using the processed products. DNA extraction was performed by using the commercial kit from Neogen Europe Ltd., UK and following the manufacturer's instructions. The DNA extraction was carried out in duplicate for each sample tested. PCR-based detection was performed by using PK mastermix/control POD from Neogen Europe Ltd., UK, and porcine and bovine specific primers from Food and Agricultural Materials Inspection Center (FAMIC), Japan.

Among the four products tested, in this study, we found out at least three were porcine DNA positive (one marshmallow, one jelly, one functional cosmetic). These result surprised us since all these products were sourced from a strictly Halal-regulated Muslim dominated country though these were imported from other countries. Moreover, one product (functional cosmetic) claimed that it contained fish collagen from Japan, which we found porcine positive.

Our results from this study proved that selection of the right primer is critical to obtain the authentic result: the porcine DNA was detected positive only when the FAMIC primer (mitochondrial DNA) was used and, in contrast, the result was negative using the NEOGEN kit primer (genomic DNA). This might be due to the higher sensitivity of mitochondrial gene target primer, which is a multicopy gene. In another study, we attempted to develop a rapid method for meat species identification based on the loop mediated isothermal amplification and electrochemical DNA sensor (Ahmed *et al*., 2010).

In a separate effort, a protein-based-immuno-chromatographic porcine detection kit was successfully co-developed under a collaborative effort between two universities (in USA and Japan) and two companies in Japan (the methods and results were not available for this paper). This development was owned and sponsored by a Japanese company having a leading position in the precious metal business including the expertise in nano-gold technology. This company obtained the immune-

chromatographic know-how from a university in Japan. However, to develop a porcine detection kit, the missing part was the biotechnology: a monoclonal antibody to a porcine thermostable muscle protein for detection of pork in cooked meat products. To address this issue, the author of this paper was approached by the Japanese company who successfully made the connection with a university in USA already reported on the availability of this antibody and he coordinated the collaboration between the Japanese company and the US University. Finally, the effort was paid-off. About 2 years later, the Japanese company confirmed the successful development of porcine detection kit and announced it. Immediately, they were approached by a large US company (renowned analytical equipment manufacturer) for this technology, which resulted in to the successful commercialization of this protein-based (immuno-chromatographic) porcine detection kit in the global market.

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